

**REGULATION OF
PARATHYROID HORMONE GENE EXPRESSION
BY
CALCIUM AND 1,25-DIHYDROXYVITAMIN D₃**

A thesis submitted to the University of London
for the degree of Doctor of Philosophy by

NIGAR SULTANA HAWA

University College London
Gower Street
London WC1

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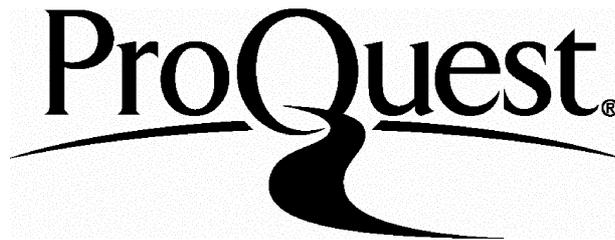
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ABSTRACT

The two major regulators of parathyroid hormone (PTH) synthesis are calcium and the steroid hormone, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and the aim of this study was to investigate the molecular mechanisms of regulation by these modulators.

Low extracellular calcium (0.4 mM) had no effect on steady-state preproPTH mRNA levels, but increased preproPTH mRNA levels associated with membrane-bound polysomes by 200%. Actinomycin D did not abolish this rise in polysomal preproPTH mRNA but increased mRNA levels by 1.6-fold in cells incubated in 0.4 and 1.0 mM calcium. Sucrose density gradient ultracentrifugation demonstrated that low calcium had no effect on polysomal size and, in addition, there was no evidence of a pool of non-ribosomal preproPTH mRNA. These data indicate that low calcium regulates PTH synthesis post-transcriptionally possibly by increasing the apparent half-life of preproPTH mRNA on polysomes.

The binding sites for the vitamin D₃ receptor (VDR) were localised using Southwestern and gel mobility shift assays which indicated two binding sites within the -451 to -348 bp and -668 to -452 bp fragments of the bovine PTH gene. To investigate functional activity of these putative VDRE, plasmids containing fragments of the -668 to +50 bp region were linked to the reporter gene, chloramphenicol acetyltransferase (CAT). The effect of 1,25(OH)₂D₃ on these constructs was investigated by transient transfection of opossum kidney cells. 1,25(OH)₂D₃ suppressed CAT activity of the construct containing the -668 to +50 bp fragment by 22%, of the -668 to -452 bp

construct by 27% and of the -451 to -348 bp construct was reduced by 25%. However, $1,25(\text{OH})_2\text{D}_3$ did not affect the activity of the construct containing the -347 to +50 bp fragment. These functional assays confirm the presence of two binding sites within the region -485 to -348 bp.

The study presented demonstrated that low calcium regulates PTH synthesis post-transcriptionally and VDR binding sites were localised on the bovine PTH gene.

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**THIS THESIS IS DEDICATED
TO MY LATE FATHER**

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CHAPTER 1
INTRODUCTION

The main work presented in this thesis is concerned with regulation of parathyroid hormone (PTH) gene expression by calcium and the active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) at the levels of transcription and translation. Thus, it is appropriate in this introductory chapter to consider the physiological role of PTH, the development of assays for the measurement of PTH, its synthesis, and the modulators of PTH secretion. In addition, an outline of protein synthesis and some of the control mechanisms which may be involved in the regulation of gene expression are presented.

Calcium homeostasis is modulated primarily by PTH and the steroid hormone, 1,25(OH)₂D₃. The maintenance of extracellular calcium within a narrow range (2.2 to 2.55 mmol/l) is important since a number of metabolic processes are influenced by small changes in extracellular ionised calcium concentrations. These include the excitability of nerve function and neural transmission, muscle cell contraction, mineralisation of newly-formed bone, blood coagulation, cell proliferation, and cytoplasmic enzyme activities.

Physiological role of PTH

PTH exerts its effects directly or indirectly on three targets: kidney, intestine and bone as shown in figure 1.1 and thereby raises calcium levels in the extracellular fluid (ECF). One of the effects of PTH is on the kidney to increase the reabsorption of calcium from the distal tubules and inhibit tubular reabsorption of phosphate and bicarbonate reabsorption from the glomerular filtrate in the proximal tubules. Furthermore, PTH has an indirect effect on

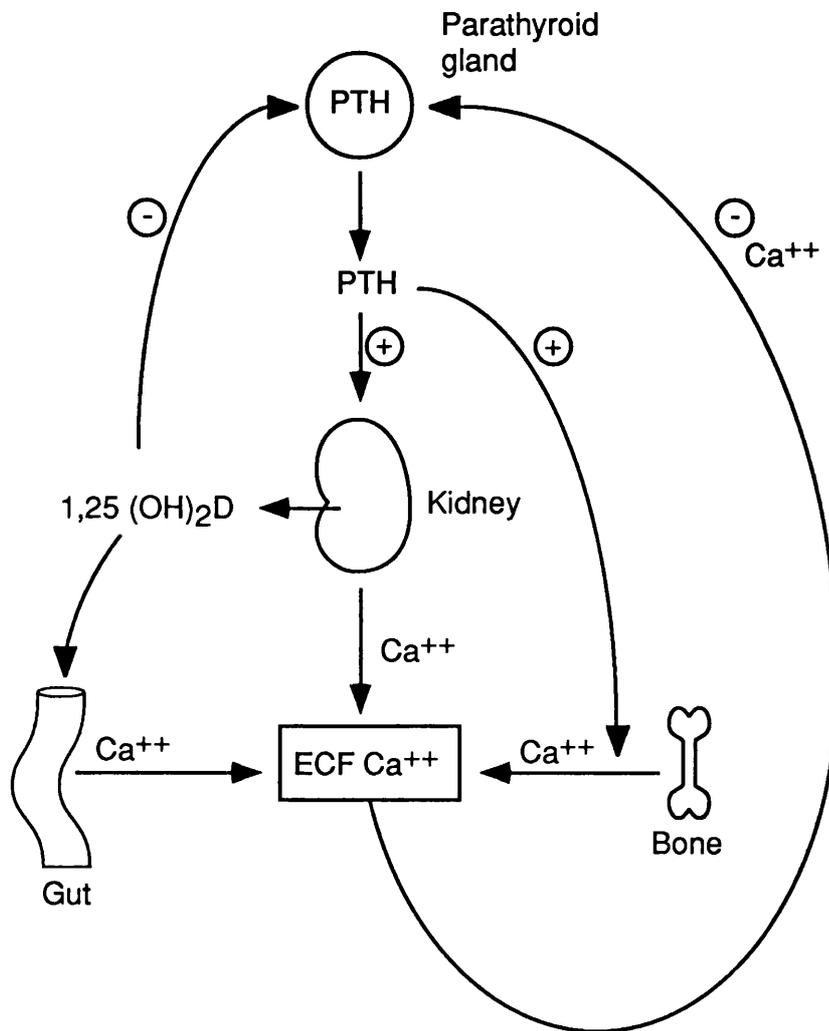


Figure 1.1. Schematic diagram showing the sites of action of PTH which are bone, kidney, and the gut. The principal action of the hormone is to raise calcium levels in the extracellular fluid (ECF) by mobilising calcium from bone, increasing renal tubular reabsorption of calcium, and increasing intestinal calcium absorption indirectly by the hydroxylation of 25-hydrocholecalciferol in the kidney. PTH levels in turn are regulated by calcium and 1,25(OH)₂D₃. The + indicates positive effects of PTH whilst the - indicates negative feedback on the parathyroid gland. This figure has been adapted from Habener *et al.* (1984).

the gastrointestinal tract through the renal 1- α -hydroxylation of 25-dihydroxyvitamin D₃ to 1,25(OH)₂D₃ (DeLuca & Holick, 1979) which is a potent inducer of intestinal calcium absorption. PTH affects bone turnover, increasing rates of bone resorption by increasing osteoclast number and activity (Raisz, 1965) and also stimulating new bone formation by osteoblasts. However, PTH appears not to have a direct effect on isolated osteoclasts (McSheehy & Chambers, 1986), but only when mixed with osteoblasts suggesting the involvement of cell-cell interactions. It appears that osteoblasts release a soluble factor after exposure to PTH which then stimulates osteoclastic bone resorption and a search for the molecule(s) responsible is currently underway. All these actions increase extracellular calcium levels, which in turn feed back on the parathyroid glands to inhibit further secretion of PTH. In addition, 1,25(OH)₂D₃ acts on the parathyroid gland inhibiting PTH synthesis and thus, keeping the calcium concentrations in the ECF within very narrow limits.

PTH binds to specific receptors (Potts et al. 1982) which are membrane-spanning glycosylated proteins of approximately 600 amino acids and are found in a number of tissues such as the kidney, bone, liver, brain, and placenta. These receptors are members of the G-protein-linked receptor family and are shared by a recently discovered factor, PTH-related peptide, (PTHrP) produced by certain tumours that cause humorally-mediated hypercalcaemia of malignancy (Broadus et al. 1985). The DNA encoding rat, opossum, and human PTH/PTHrP receptors has recently been cloned and enabled the sequence to be deduced (Juppner et al. 1991; Abou-Samra et al.

1992). Binding of PTH to its receptor on the plasma membrane activates G proteins which are composed of three subunits, α , β and γ on the inner surface of the membrane causing the release of guanosine diphosphate (GDP) from G_{α} . This results in the dissociation of the G_{α} from $G_{\beta,\gamma}$ subunits and permits the binding of a molecule of guanosine triphosphate (GTP) to G_{α} . The GTP - G_{α} complex then activates membrane-bound adenylate cyclase to catalyse the synthesis of cyclic 3',5'-adenosine monophosphate from ATP. cAMP subsequently binds to the regulatory subunit of cAMP-dependent protein kinase A causing the regulatory subunit to dissociate from the catalytic subunit of protein kinase. The free catalytic subunit then phosphorylates specific serine and threonine residues in target proteins for example, enzymes responsible for ion transport.

In order to maintain ionised calcium levels within a narrow range, it is essential that PTH and $1,25(\text{OH})_2\text{D}_3$ levels are controlled. Excessive PTH causes hypercalcaemia and phosphaturia which are most commonly due to a benign tumour, an adenoma, of one of the four parathyroid glands. This condition is called primary hyperparathyroidism and is characterised by bone loss due to osteoclastic resorption of bone and renal calculi. However, hypercalcaemia and phosphaturia can also be due to hyperplasia or carcinoma. Secondary hyperparathyroidism occurs due to overactivity of the glands compensating for long-standing hypocalcaemia which can be caused, for example, by chronic renal failure. Hypercalcaemia is also seen in non-parathyroid malignancy, humoral hypercalcaemia of malignancy (HHM), due to the production of circulating humoral factors such as PTHrP that cause

hypercalcaemia (Stewart *et al.* 1980). The malignancies frequently associated with HHM include squamous cell carcinoma of the lungs, neck and head, carcinoma of the kidney, ovary and pancreas. The presence of these tumours is associated with an increase in osteoclastic bone resorption, an increase in renal tubular reabsorption, and impaired calcium absorption from the gut. Secretion of PTH from these tumours is rare and this malignancy is characterised by production of a protein, PTHrP which has been purified (Burtis *et al.* 1987; Moseley *et al.* 1987) and consequently its cDNA was cloned (Suva *et al.* 1987; Mangin *et al.* 1989). Low PTH (hypoparathyroidism) may be due to diseases in the parathyroid gland causing impaired synthesis and secretion of PTH and is characterised by hypocalcaemia and hyperphosphataemia. In addition, it may be due to target organ resistance to PTH resulting in a condition called pseudohypoparathyroidism which is characterised by hypocalcaemia and high PTH levels. These patients are unresponsive to PTH despite high circulating levels of PTH and the defect may lie in the PTH receptors.

The biologically active vitamin D metabolite, $1,25(\text{OH})_2\text{D}_3$, is synthesised in the kidney by hydroxylation of 25-hydroxyvitamin D_3 (synthesis of $1,25(\text{OH})_2\text{D}_3$ is described further in chapter 3). Its primary function is to enhance calcium absorption from the small intestine and during vitamin D deficiency, intestinal calcium and phosphate absorption are reduced, causing hypocalcaemia which leads to defective bone mineralisation. Deficiency of vitamin D causes rickets in children; in adults the corresponding condition is osteomalacia. Both conditions are characterised by the failure of calcification

of osteoid, which is the matrix of bone and the manifestations depend on whether or not the bone is growing. The serum calcium is low and may lead to tetany and increases parathyroid gland activity. Deficiency of vitamin D in the UK occurs in two groups of the population, the elderly and the Asian immigrants. The deficiency may occur due to a number of causes, including diet and lack of exposure to sunlight. There are other causes of rickets and osteomalacia apart from a deficiency of vitamin D; chronic renal failure is one cause and this is probably because there is a lack of the 1- α -hydroxylase activity with the loss of functioning renal tissue.

Assays for the measurement of PTH

During the 1960s and 1970s, substantial progress was made in the understanding of the biochemical and cellular processes involved in the biosynthesis, secretion, metabolism and mode of action of PTH. These advances have been due to the application of radioimmunoassay (RIA) techniques developed for the detection of PTH. A sensitive RIA for bovine PTH was first reported by Berson *et al.* (1963) using an anti-bovine PTH antiserum, ¹³¹I-labelled bovine PTH as labelled antigen and human PTH extracted from adenoma as a standard. These RIA were used to detect endogenous human PTH in plasma samples obtained from two patients with primary hyperparathyroidism and in normal subjects; the hormone was undetectable in the plasma of nine patients with clinical hypoparathyroidism. Extensive use has been made of this assay system in physiological studies in animals (Sherwood *et al.* 1966), but these assays were not sensitive enough

to discriminate low from normal PTH values as well as between normal and slightly elevated values. Since very little pure human parathyroid hormone was available for use in assays, antisera raised against bovine PTH which were specific and sufficiently sensitive to measure endogenous hormone in humans were used. It wasn't until later, using parathyroid tissue removed at surgery, O'Riordan et al. (1971) isolated and purified sufficient human PTH for use in RIA and for chemical characterisation.

Apart from the structural differences and cross-reactivity between PTH from bovine and human sources, the main problem in measuring absolute concentrations of hormone in the circulation was the presence of different PTH-derived peptides. The first observation suggesting the immunochemical heterogeneity of PTH in the circulation was reported by Berson & Yalow (1968) based on the measurement of the half-life of plasma immunoreactive PTH after parathyroidectomy in patients with secondary hyperparathyroidism due to chronic renal failure which was slower than in patients with primary hyperparathyroidism. These differences were demonstrated using two distinct antisera indicating that PTH in human plasma was immunochemically heterogeneous and that removal of PTH fragments from the circulation was probably dependent on renal function. A number of PTH fragments resulting from proteolysis of the native polypeptide within the parathyroid gland and in peripheral organs (in the kidneys and liver into amino-terminal and carboxy-terminal fragments) are found in the circulation (Canterbury et al. 1975). In addition, parathyroid glands contain cathepsins that are responsible for further cleavage of PTH into smaller fragments which are secreted (MacGregor et al.

1979; Morrissey *et al.* 1980). In normal humans, intact PTH comprises only 5-30% of the circulating immunoreactive hormone, whilst the C-terminal fragments account for the remaining 70-95%. Thus, the importance of developing assays for the detection of defined regions of PTH and the application of these assays to the measurement of the various fragments in the circulation becomes apparent. This eliminated the difficulty in interpretation of results obtained with different RIAs for PTH in the clinical assessment of patients.

Region-specific RIA were thus developed for measuring C-terminal fragments i.e. those which detect the intact hormone and circulating carboxy-terminal fragments, and for measuring N-terminal fragments which detect the intact hormone as well as any circulating amino-terminal fragments. However, several problems have been encountered, including the susceptibility of PTH to damage during radiolabelling and the deterioration of labelled preparations during the assay which may affect the affinity of the antisera. In order to overcome some of these difficulties, immunoradiometric assays (IRMA) were developed (Addison *et al.* 1971). In contrast to RIA in which labelled and unlabelled antigen compete for binding to specific antibodies, in IRMAs, the compound to be measured is assayed directly by combination with specifically labelled antibodies. This method has advantages with regard to sensitivity and precision and in addition, the labelled antibody preparation is more stable. IRMA specific for the amino-terminal (Manning *et al.* 1980) or carboxy-terminal (Manning *et al.* 1981) regions have been developed using immunoabsorbents consisting of fragments of human PTH 1-34 or 53-84

coupled to cellulose. The assay was used to measure carboxy-terminal immunoreactive PTH in normal patients, as well as those with primary hyperparathyroidism, pseudohypoparathyroidism and secondary hyperparathyroidism. It was of diagnostic value for assessing patients with chronic hyperparathyroidism since the authors found good discrimination between normal subjects and patients with primary hyperparathyroidism. A two-site IRMA for intact PTH was subsequently developed (Nussbaum et al. 1987), utilising two different antibodies to PTH, mid-region/C-terminal and N-terminal. The mid-region/C-terminal antisera is bound to a solid phase and is present in excess, whilst the N-terminal antisera is radiolabelled (^{125}I) and used as a tracer. This assay showed discrimination of PTH values in patients with primary hyperparathyroidism from those with hypercalcaemia associated with cancer, as well as between normals and patients with hypoparathyroidism. Other methods have been described for the measurement of PTH in serum or plasma such as cytochemical bioassays (Chambers et al. 1978). Although these assays are very sensitive, they are not used routinely for clinical diagnosis since the number of samples that can be analysed is limited.

In order to produce antisera against PTH, it was essential that the peptides were isolated, purified for immunisation and their structures determined. The primary structures of the 84 amino acid polypeptides of the porcine (Sauer et al. 1974), bovine (Niall et al. 1970) and human (1-34 amino acids by Brewer et al. 1972; amino terminal 37 residues by Niall et al. 1974; residues 44-84 by Keutmann et al. 1977) PTH have been determined. The determination of

the amino-terminal residues of hPTH has enabled the synthesis of peptide fragments and analogues, including antagonists and agonists of PTH for clinical and investigative use. In addition, these synthetic fragments have enabled the development of antisera directed towards the amino-terminal region of the hormone and used for measuring PTH in sera from patients with primary hyperparathyroidism and in normal subjects (Hendy et al. 1974). The amino-terminal sequences of human PTH extracted from human adenoma determined by Brewer et al. (1972) and Niall et al. (1974) differed at three positions in the first 30 residues and on re-examination of the sequences each confirmed their own findings. Further studies using RIA specific to the amino-terminal region of PTH were used to study the properties of synthetic peptides based on the structures proposed by Brewer et al. (1972) and Niall et al. (1974) compared to extracted human PTH (1-84) from human parathyroid adenomas (Hendy et al. 1974). These assays revealed that the peptides based on the structure proposed by Brewer et al. (1972) were less reactive (100-to 2000-fold) and thus it appears that since the peptides with the sequence proposed by Niall et al. (1974) behave similarly to purified hPTH (1-84), this sequence is more likely to be correct. Subsequently, mRNA nucleotide sequence analysis has confirmed the sequence of the former group (Hendy et al. 1981).

Determination of PTH gene sequences

Recombinant DNA techniques have enabled the isolation and cloning of complementary DNA (cDNA) to bovine (Kronenberg et al. 1979) and human

(Hendy et al. 1981) preproPTH mRNA in bacterial hosts. Bovine mRNA was isolated from normal parathyroid glands, human mRNA was isolated from parathyroid adenomas and the sequence of the rat mRNA was established from rat liver (Heinrich et al. 1984). Kronenberg et al. (1979) initially determined the sequence of a bovine cDNA clone, pPTHm1, that contained about 60% of the PTHmRNA, including all of the region coding for preproPTH plus portions of the 5' and 3' noncoding regions of the mRNA. Restriction analysis of near full-length double-stranded cDNA, synthesized enzymatically from partially purified bovine PTH mRNA, indicated that about 200 nucleotides from the 3'-untranslated region were missing in the clone (Gordon & Kemper, 1980). A near full-length clone of bovine PTH mRNA, pPTHi4, was later sequenced (Weaver et al. 1984) and this showed that the overlapping sequences of pPTHm1 and pPTHi4 were identical except for the first 50 nucleotides. Analysis of several additional bovine PTH cDNA clones and the sequencing of the 5' terminus of PTH mRNA indicated that the first 50 nucleotides of pPTHm1 were an inverted complement of the corresponding sequence of pPTHi4 (Weaver et al. 1981). This may have occurred in the process of cloning; the single strand of the cDNA may have folded back on itself during reverse transcription or during the synthesis of the second strand by DNA polymerase. cDNA probes have facilitated the study of PTH biosynthesis proving useful as hybridisation probes for the measurement of PTH mRNA levels and as probes in screening libraries by filter hybridisation in the cloning of PTH genes.

The gene for PTH is located on chromosome 11 (Zabel et al. 1985) and is

represented only once in the haploid genome of humans (Vasicek et al. 1983), rats (Heinrich et al. 1984) and cows (Weaver et al. 1984). In all species, the PTH gene consist of three exons, the 5'- and 3'-flanking regions and two intervening sequences (introns) and the structure of the gene is described further in chapter 2.

Synthesis of PTH

The pathway leading to PTH secretion involves a series of proteolytic cleavages of a large precursor (Cohn & Elting, 1983). The primary translation product synthesized in the parathyroid gland is a polypeptide of 115 amino acids, preproparathyroid hormone (preproPTH). This is cleaved by a signal peptidase located on the inner membrane of the endoplasmic reticulum (ER) (Habener et al. 1975) which removes the signal or 'pre' sequence as the protein traverses the membrane of the rough ER, leaving proPTH (90 amino acids) in the cisternae of the ER (Habener et al. 1978). This cleavage is very rapid and very little intact preproPTH can be found in parathyroid cells since preproPTH has a half-life of 15 minutes. The 'pro' sequence is cleaved from proPTH in the Golgi apparatus by a trypsin-like endopeptidase, followed by carboxypeptidase B (Habener et al. 1977) resulting in bioactive PTH. The 'pro' sequence is subsequently degraded in the parathyroid cell after cleavage from PTH. The mature hormone is packaged into secretory granules and stored prior to secretion at which time the membranes of the storage granules fuse with the plasma membrane, membrane lysis occurs and the contents of the vesicle are released into the bloodstream. However, some PTH can be

transported directly to the plasma membrane without incorporation into mature secretory granules (MacGregor et al. 1975).

The parathyroid gland synthesises another major protein, parathyroid secretory protein (PSP) or chromogranin A (CgA) which co-exists in the same secretory granules (Cohn et al. 1982). This is an acidic glycoprotein found in endocrine cells and co-secreted with the resident hormone (Winkler & Fischer-Colbrie, 1992). It is possible that CgA serves as a precursor to bioactive peptides that modulate secretion in an autocrine and/or a paracrine manner (Huttner & Benedum, 1987; Eiden, 1987) and that regulation of CgA expression and processing is tissue-specific (Barbosa et al. 1991; Defetos et al. 1990). The secretion of CgA like PTH is regulated by extracellular concentrations of calcium, such that low calcium stimulates, and high calcium inhibits secretion (Morrissey & Cohn, 1979).

Modulators of PTH secretion

The availability of cDNA probes for preproPTH mRNA has made studies possible on the control of synthesis and secretion of PTH. Calcium has been shown to regulate PTH gene expression both in vitro (Russell et al. 1983; Brookman et al. 1986) and in vivo (Yamamoto et al. 1989; Naveh-Many et al. 1989). Short-term experiments (4-7 hours) in isolated bovine parathyroid cells have shown that high extracellular calcium caused changes in PTH secretion with no detectable change in preproPTH mRNA levels (Heinrich et al. 1983; Brookman et al. 1986), whilst longer incubation periods of 16-24 hours resulted in a decrease in mRNA levels with a concomitant fall in

secretion (Russell et al. 1983; Brookman et al. 1986). In addition, studies in cultured human adenomas incubated with high calcium have demonstrated a similar reduction in preproPTH mRNA levels as in normal bovine glands. However, the continued synthesis of PTH in adenomas compared with the decrease in bovine glands, implies that regulation may also be post-transcriptional (Farrow et al. 1988). Incubation of bovine parathyroid cells in vitro with low calcium resulted in an increase in PTH synthesis with no change in steady-state preproPTH mRNA levels, also suggesting the existence of a post-transcriptional site of regulation. The effects of low calcium on PTH secretion and the possible regulatory mechanisms will be discussed in the following chapter.

The mechanism whereby calcium regulates secretion and transcription in the parathyroid cell remains to be determined. Okazaki et al. (1991) have presented results of transfection studies in which they have identified two negative regulatory elements between 2.4 and 3.6 kb upstream of the transcription start site of the human PTH gene and have shown that these DNA sequences bind nuclear proteins resulting in suppression of transcription. The authors have postulated that these negative regulatory elements may be responsible for modulation of transcriptional suppression of the hPTH gene by extracellular calcium. Although, calcium is the major modulator of PTH secretion, other agents have been identified such as magnesium (Habener & Potts, 1976) which causes similar changes as calcium, however, magnesium is approximately 2.5-times less effective on a molar basis than calcium in altering the rates of PTH secretion in vitro.

Another important regulator of PTH synthesis is $1,25(\text{OH})_2\text{D}_3$ and studies in vitro and in vivo have demonstrated that $1,25(\text{OH})_2\text{D}_3$ decreases PTH synthesis (Cantley et al. 1985; Silver et al. 1985, Karmali et al. 1989), whilst Sherwood et al. (1987) have shown that this is a direct effect on PTH gene transcription. $1,25(\text{OH})_2\text{D}_3$ acts on its target tissue by binding to its receptors, vitamin D_3 receptors (VDR), with binding to specific DNA sequences of target genes subsequently affecting gene transcription (this will be discussed further in chapter 3). Glucocorticoids such as dexamethasone have been shown to stimulate PTH in a dose-dependent manner (Sugimoto et al. 1989) and, in addition, cortisol has been shown to produce elevated levels of serum PTH when administered in humans (Fucik et al. 1975). It has been speculated that cortisol may lower serum calcium levels by reducing intestinal absorption and renal tubular resorption of calcium. However, Au, (1976) has reported a direct stimulatory effect of cortisol on the secretion of PTH from rat parathyroid glands maintained as explants in culture, which suggests mechanisms independent of changes in blood levels of calcium. Karmali et al. (1989) have shown that in cultured bovine parathyroid cells, the suppressive effect of $1,25(\text{OH})_2\text{D}_3$ on PTH gene transcription was abolished in the presence of cortisol i.e. PTH mRNA levels and secretion remained unchanged. This effect was reversible, in that withdrawal of cortisol resulted in the return of the response to $1,25(\text{OH})_2\text{D}_3$. The effect of cortisol on preventing this response was at least partly mediated by reducing the number of VDR and thus possibly reducing the sensitivity to $1,25(\text{OH})_2\text{D}_3$. Other potential regulators include oestradiol and progesterone and studies on isolated

bovine parathyroid tissue suggest that both these hormones directly stimulate PTH secretion (Greenberg et al. 1987). This study demonstrated that the effect of the two steroids was stereospecific since 17 α -oestradiol and 20 α -hydroxyprogesterone had no effect. The mechanism of oestradiol is not clear, however, it has been suggested that receptors may not be involved since the antagonist, tamoxifen, did not prevent this increase. In addition, Naveh-Many et al. (1992) have shown that 17 β -oestradiol given to ovariectomised rats led to an increase in PTH gene expression and that this was a direct effect on the parathyroid glands. Their results appear to show that the parathyroid glands are target organs for oestrogen leading to increased expression of PTH which may be helpful in counteracting or preventing bone loss.

PROTEIN SYNTHESIS

It is appropriate at this stage to give an outline of protein synthesis and the regulatory control mechanisms of gene expression as the main theme of the studies presented here is regulation of PTH gene expression at the transcriptional and post-transcriptional levels.

Synthesis of mRNA or transcription is the result of RNA polymerase II initiation at a promoter sequence and involves the interaction of several general transcription factors (TF) such as TFIID which recognises the TATA sequence, and TFIIA, TFIIB and TFIIE which assemble sequentially into a transcription complex (Matsui et al. 1980; Tsai et al. 1981; Dynan & Tjian, 1983). An initial interaction of TFIID and TFIIA with the TATA-box element of a promoter gives rise to a template-committed complex (Reinberg et al.

1987). This complex provides an anchor for subsequent assembly of other transcription factors including TFIIE and TFIIIB and RNA polymerase II into a complete initiation complex (rapid start complex).

Following transcription, the nucleotide sequence of RNA is translated into amino acids and this occurs in three stages, initiation, elongation and termination. The first event of the initiation stage involves the interaction of the 40S ribosomal subunit, mRNA, and initiation factors with GTP and a molecule of methionyl-tRNA_i^{Met} to form an initiation complex. This complex migrates linearly until it reaches the AUG initiation codon whereupon a large ribosomal subunit (60S) joins this complex, resulting in hydrolysis of GTP and detachment of some initiation factors. There are two sites on the ribosome that are occupied by tRNA molecules, the A and P site, and the methionyl-tRNA_i^{Met} bearing the first amino acid binds to the P site whilst the A site accommodates the incoming aminoacyl-tRNA. The amino acid is transferred to the P site after the formation of a peptide bond, resulting in a growing polypeptide chain. Several elongation factors are involved and GTP provides the energy to position the aminoacyl-tRNA in the A site. Translation is terminated when the ribosome reaches the UAG codon, recognised by termination factors, followed by hydrolysis of the peptidyl-tRNA on the ribosome and the release of the completed polypeptide chain. Subsequently, the tRNA and the two ribosomal subunits separate from the mRNA. Once ribosomes have translated mRNA, they preferentially and efficiently reinitiate protein synthesis on the same mRNA resulting in polyribosomes or polysomes.

The synthesis of proteins destined for secretion such as PTH, insulin and

prolactin takes place on ribosomes bound to the membrane of the rough endoplasmic reticulum (RER) (Palade, 1975), whilst intracellular proteins such as the glycolytic enzymes are translated preferentially on free ribosomes. The 'pre' or signal sequence of secretory proteins has been shown to direct the protein to the RER (Blobel, 1983). As the signal sequence emerges from the ribosome, it binds to the signal recognition particle (SRP) and this complex binds the ER membrane through the SRP receptor (Meyer et al. 1982). The receptor is an integral protein of the ER and this binding is associated with the release of the SRP into the cytosol. The signal sequence is cleaved in the ER lumen by signal peptidase which may cause the receptor to be released from the signal sequence. Elongation of the peptide chain continues and it extrudes into the lumen where it undergoes post-translational modifications such as folding, glycosylation, sulphation and phosphorylation. Glycosylation is the major modification to proteins as they pass through the Golgi apparatus and involves the addition of oligosaccharide groups either to the amino group of asparagine (N-linked glycosylation) or to the hydroxyl group of serine, threonine or hydroxylysine (O-linked glycosylation). Secretory proteins are directed to secretory vesicles where they are stored before exocytosis and often undergo proteolytic cleavage, for example, hormones such as insulin are synthesised as a large precursor which is cleaved to generate the active hormone (Steiner et al. 1967). Some modifications to proteins occur after secretion, for example, tropocollagen helices lose the N- and C-terminal prosequences before being incorporated into collagen fibres (Davidson & Berg, 1981). In addition, activation of enzymes such as trypsinogen and

chymotrypsin also occurs by specific proteolytic cleavages after secretion.

CONTROL OF GENE EXPRESSION

Gene expression can be regulated at multiple levels including initiation, elongation or termination of transcription and post-transcriptionally which includes a number of mechanisms such as nuclear processing of primary transcripts and mRNA stability. Each of these will be discussed further, with a variety of examples.

Transcriptional control

Initiation

Regulation at the transcriptional level involves interaction of specific regulatory sequences and trans-acting proteins which bind DNA sequences in the promoter region of genes (Dyana & Tjian, 1985). Promoter sequences lie within approximately 100 base pairs (bp) of the start site of transcription and act to increase or decrease the rate of transcription. The activity of many promoters is modulated by enhancer sequences which are a combination of discrete, closely spaced sequences found upstream or downstream that can act over long distances to stimulate transcription (Maniatis *et al.* 1987). Regulatory proteins at widely separated sites may influence the activity of another protein by looping of intervening DNA sequences, thus allowing protein-protein interactions. This has been demonstrated by Hochchild & Ptashne (1986) in studies of λ -phage repressor. Alternatively, a protein on recognising a specific site on DNA may 'move' or 'slide' along the DNA to

another specific sequence, interact with another protein and thus initiate transcription. Silencers are another class of DNA elements which may lie far upstream from the start site and inhibit transcription, often independently of their position or orientation (Brand et al. 1985). Silencer sequences have been reported in various genes, for example, α -fetoprotein (Laimins et al. 1986), growth hormone (Larsen et al. 1986) and human PTH (Okazaki et al. 1991).

Promoter elements also interact with DNA-binding proteins, such as stimulatory protein I (SPI) (Dyran & Tjian, 1983), CTF/NF-1 (Jones et al. 1987) and proteins of the AP1 family (Kouzarides & Ziff, 1988; Turner & Tjian, 1989). These factors function by protein-protein interactions with other components of the general transcription apparatus, including RNA polymerase II. Another family of DNA-binding proteins involved in transcriptional regulation are cAMP response element-binding proteins (CREB) which recognise the cAMP-response element (CRE) sequence, 5'-TGCCGTCA-3'. This motif is also recognised by activating transcription factor (ATF) proteins (Hai et al. 1989). Many promoters respond transcriptionally to elevated levels of cAMP through cAMP response elements (Montminy et al. 1986; Short et al. 1986; Silver et al. 1987) and several CREB have been characterised which are activated by cAMP-dependent protein kinase A (Hoeffler et al. 1988; Gonzalez et al. 1989). A functional CRE has been identified in the promoter region of the somatostatin (Montminy et al. 1986), c-fos (Yamamoto et al. 1988) and human PTH gene (Rupp et al. 1990).

Transcription is also influenced by diffusible substances such as steroid hormone-receptor protein complexes. For example, Bagchi et al. (1992) using

a cell-free transcription assay have shown that progesterone receptors facilitate the assembly of a template-committed complex at the TATA box and subsequently stimulate transcriptional initiation by enhancing the formation of a rapid start complex containing RNA polymerase II. Steroid hormone receptors act as ligand-inducible transcription factors which induce or repress transcription by interacting with short consensus DNA sequences, hormone response elements (HREs), located in the 5'-flanking region of genes, and trigger the general transcription machinery to assemble. The steroid hormone, 1,25(OH)₂D₃, modulates gene transcription via its receptors, vitamin D₃ receptors (VDR), which are members of the steroid/thyroid hormone receptor superfamily and interact with vitamin D₃ response elements (VDRE) on target genes.

HREs usually consist of two closely related or identical copies of a short sequence motif arranged as palindromes (inverted repeats) immediately adjacent to each other as seen in the rat growth hormone thyroid response element, (TRE pal) (Glass *et al.* 1988) or with a three nucleotide spacing as in the vitellogenin gene oestrogen response element (ERE) (Klein-Hitpass *et al.* 1986) as shown below. The DNA targets for VDR and many other nuclear receptors are often organised as direct or imperfect repeats of half-site palindromes with variable spacing as seen in the rat cardiac myosin heavy chain (MHC) TRE (Izumo & Madhavi, 1988):

<u>Hormone response element</u>	<u>Consensus sequence</u>
Thyroid response element (pal)	AGGTCATGACCT
Oestrogen response element	AGGTCA nnn TGACCT
Myosin heavy chain TRE	AGGTGA nnnn AGGACA

* n denotes nucleotide spacing between half-sites.

HREs are structurally related but functionally distinct, for example, glucocorticoid receptors (GRs) in vitro can bind to the consensus sequence AGAACA nnn TGTTCT but several other receptors such as the progesterone, mineralocorticoid and androgen receptors also bind this sequence. Likewise, the glucocorticoid response element (GRE) consensus sequence is similar to that of the oestrogen response element (ERE), however, the ERE and the GRE are functionally distinct. Thyroid hormone receptors (TR) also bind to a large family of related sequences such as the ERE in vitro (Glass et al. 1988). These studies have shown that although TR bind to both the TRE and the ERE as a dimer, the formation of a domain competent to activate transcription requires a response element without nucleotide spacing. This domain is disrupted when the TR is bound to an ERE and the protein-DNA complex fails to activate transcription. Thus, these authors suggest that the presence or absence of a 3 nucleotide gap in the DNA recognition sequence for TR can dictate negative or positive transcriptional control by thyroid hormone (T₃). Selective hormonal responsiveness is determined not only by the characteristics of a HRE but also by the presence of their respective ligands as well as the presence of other proteins or transcription factors that interact

with an element.

Elongation

Following initiation, the nascent RNA chain is elongated by the addition of ribonucleoside triphosphates catalysed by RNA polymerase II which is another site of transcriptional control. Studies by Chinsky *et al.* (1989) have demonstrated tissue-specific differences in adenosine deaminase gene expression associated with processes affecting the synthesis of full length nascent transcripts. These studies showed that during adenosine deaminase transcription, a certain portion of the nascent RNA was prevented from being synthesised into completed primary transcripts i.e. elongation was blocked. Other examples of genes in which elongation is blocked during transcription include several proto-oncogenes such as c-myb (Bender *et al.* 1987), c-fos (Fort *et al.* 1987) and c-myc (Bentley & Groudine, 1988).

Termination

Regulation at the level of termination of transcription also occurs, although there is considerable difficulty in identifying the authentic termination site of an eukaryotic gene, since it is always possible that the 3' end has been generated by cleavage of the primary transcript and, therefore, not the exact site of termination. However, the sites of transcription termination for RNA polymerase II *in vivo* have been localised to regions that lie 3' to the coding sequences and downstream of the poly (A) processing site for several genes (Sato *et al.* 1986, Proudfoot, 1989). Several groups have indicated the

presence of a series of thymidine residues (T-runs) in DNA sequences which are thought to act as termination signals and may be involved in regulation of gene expression, for example, in the human gastrin gene (Sato *et al.* 1986), and c-myc (Bentley & Groudine, 1988).

Translational control

Eukaryotic cells employ translational control mechanisms in addition to transcriptional control to regulate the synthesis of specific proteins. This is usually much more rapid than control at the level of transcription. Several possible mechanisms and sites of post-transcriptional control exist as shown in the schematic diagram (figure 1.2). Possible sites of regulation include the addition of a 7-methyl guanosine (m^7G) cap to the 5' end, polyadenylation and splicing of the heterogenous RNA in the nucleus producing mature mRNA containing the 5'- and 3'- untranslated regions as well as the coding regions (step 1). The mature mRNA is subsequently transported out into the cytoplasm where it may be degraded (step 2) without being translated (step 3). Alternatively, it may interact with proteins forming messenger ribonucleoprotein particles (mRNPs) which may exist in an untranslatable form (step 4). In this case, a suitable stimulus may cause this pool of mRNA to be released and available for translation, thus increasing translation without altering steady-state mRNA levels. Increases in protein synthesis may also be due to an increased rate or efficiency of translation i.e an increase in initiation and an increase in elongation or an increase in reinitiation relative to the rate of elongation of polypeptide chains on polysomes (step 5).

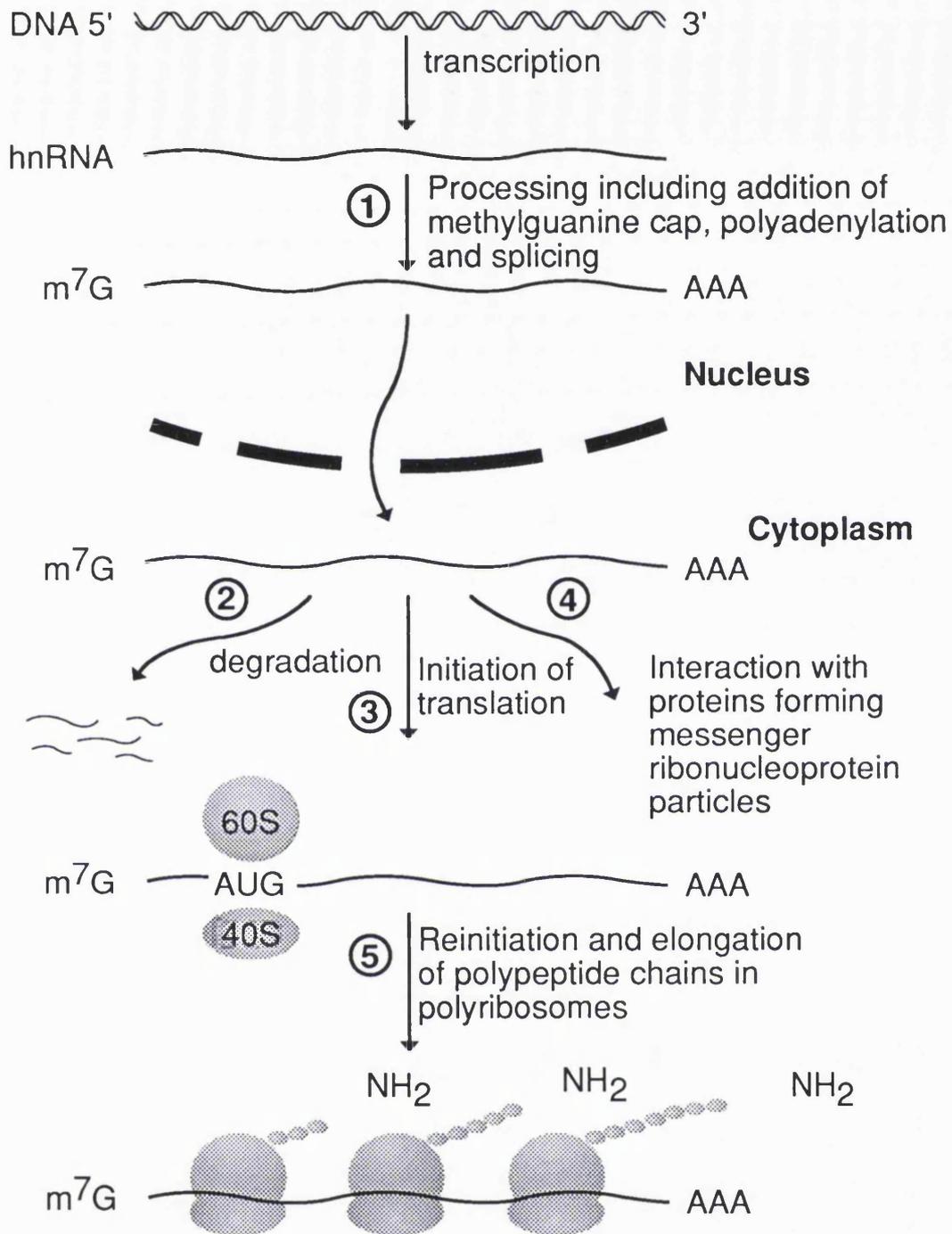


Figure 1.2. Pathway of protein synthesis from gene transcription to polypeptide synthesis. Indicated are the possible sites of regulation including processing of heterogeneous RNA (step 1), transport of mRNA into the cytoplasm where it may be degraded (step 2) without being translated (step 3) or interaction with proteins to form messenger ribonucleoprotein particles (step 4). Increases in protein synthesis may also be due to changes in reinitiation and elongation of polypeptide chains in polysomes (step 5).

The possible sites of regulation can include most of these steps and will be discussed in more detail below under the headings of nuclear processing of primary transcripts, regulation of mRNA stability, presence of an inactive pool of mRNA, and changes in the rate of initiation/reinitiation and rates of elongation of polypeptide chains.

Nuclear processing of primary transcripts

Differential processing of heterogeneous nuclear RNA (hnRNA) such as addition of a cap structure or polyadenosine (poly(A)) residues and splicing of introns provides another mechanism of translational control in eukaryotes. Capping of mRNA is a process that occurs almost immediately after transcription and involves joining of a residue of 7-methylguanosine (m^7G) by a 5'-5' pyrophosphate linkage to the initial nucleotide at the 5' end of mRNA. These caps contribute to the stability of mRNAs by protecting the 5' ends from phosphatases and nucleases and probably facilitate ribosome attachment to mRNA.

The protein-coding regions of eukaryotic genes are split by intervening sequences (introns) which are removed from the initial transcript by splicing to give mature mRNA containing the 5'- and 3'-untranslated regions (UTRs) in addition to those sequences which are translated. Splicing occurs exclusively in the nucleus during or after polyadenylation and involves breaking the phosphodiester bonds at the exon-intron boundaries and forming a bond between the ends of the exons. RNA transcripts can also be spliced alternatively in several different tissues resulting in different functional

mRNAs and are translated to yield different protein products. Alternative splicing represents a response to a requirement for the production of related, but different forms, of a gene product in different tissues as seen in the opioid peptide genes (Garret *et al.* 1989) and the calcitonin/calcitonin gene related peptide gene (Amara *et al.* 1982).

Several hypotheses have implicated poly(A) sequences as having a role in mRNA stability, protein synthesis and the transport of mRNA out of the nucleus. Immediately after splicing of the hnRNA primary transcript, nuclear poly(A) polymerase adds 150-200 nucleotides to primary transcripts that have a 3' terminal GC and an AAUAAA sequence 15-30 residues from the 3' end. The turnover of mRNA is often related to the presence or absence of poly(A) tails, for example, stabilisation of human growth hormone mRNA by glucocorticoids appears to be due to an increase in the length of their poly(A) tail which is reduced when the mRNA is destabilised by removal of glucocorticoids (Paek & Axel, 1987). Binding of a poly(A)-binding protein is thought to be required for protection of the 3' end of the mRNA and when the length of poly(A) tract falls below 20-30 nucleotides, the protein may not bind, resulting in rapid degradation. The role of the poly(A) tail in protein synthesis has been demonstrated by Rubin & Halim (1993), in which they showed that the poly(A) tail of globin mRNA shortened during translation. The addition of aurintricarboxylic acid (ATA), which prevents the binding of mRNA to the 40S ribosomal subunit, to the components of the translation reaction mixture resulted in very little incorporation of radiolabelled leucine into globin and shortening of the poly(A) tail almost ceased when compared

to the reaction in the absence of ATA. These authors suggest that the poly(A) tail in translation is to possibly bring the 3'-end in close proximity to the 5'-end, thus facilitating reinitiation.

Rat pituitary acidophil cells consist of lactosomatomorphs which produce growth hormone (GH) and prolactin (PRL) (GH+/PRL+), somatomorphs which produce GH but not PRL (GH+/PRL-) and lactomorphs which produce PRL only (GH-/PRL+). Studies in a series of pituitary cell lines which are either GH+/PRL+ or GH+/PRL- have demonstrated that transcription rate of the PRL gene in the latter was 60-70% of that in the former, but cytoplasmic mRNA levels were only 2% (Billis *et al.* 1992). This was shown to be due to PRL gene transcripts being specifically degraded in the nucleus rather than being processed and exported into the cytoplasm.

Regulation of mRNA stability

Changes in the rate of degradation of mRNA is another mechanism of regulation. Certain sequences, AUUUA, in the 3'UTR are involved in the degradation of mRNA. The 3'-UTR of the human granulocyte-monocyte colony-stimulating factor (GMCSF) mRNA contains seven AUUUA sequences resulting in a short half-life. This was demonstrated when a segment of the human GMCSF DNA containing these sequences was inserted into the 3'UTR of the rabbit β -globin gene and decreased the half-life of the β -globin mRNA from 2 hours to less than 30 minutes (Shaw & Kamen, 1986). Stability of specific mRNAs may be affected by hormones such as glucocorticoids, for example, Diamond & Goodman (1985) reported that dexamethasone and

thyroid hormone (T_3) increased the stability of growth hormone mRNA in pituitary cells. Glucocorticoids have also been shown to decrease the half-life of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase mRNA in rat liver, whilst thyroid hormone had the opposite effect (Simonett & Ness, 1989). In the presence of dexamethasone, the half-life of the transcripts was reduced from 12-15 hours to 2-3 hours, suggesting that dexamethasone may stimulate degradation of HMG-CoA reductase mRNA. Glucocorticoids may induce the expression of an enzyme that degrades mRNA which contain AUUUA sequences or alternatively, they may counteract a thyroid-induced protein which stabilises the mRNA. In contrast, Krane *et al.* (1991) have reported that in rat pituitary cells, thyroid hormone (T_3) reduced the size and stability of the TSH β -subunit mRNA and this reduction in size was due to shortening of the poly(A) tail.

Presence of an inactive pool of mRNA

The recruitment of mRNA from a storage form (untranslatable) in response to a specific stimulus provides another example of translational control. The existence of an inactive pool of mRNA has been well-documented with respect to ferritin synthesis. Iron administration to rats increases hepatic ferritin synthesis but has little effect on ferritin gene transcription (Aziz & Munro, 1986). Following iron administration, ferritin mRNA was rapidly recruited from an 'untranslatable' pool in the cytoplasm bound to mRNP or other proteins onto polysomes for active translation. This mechanism provides a rapid response without altering gene transcription or steady-state mRNA

levels.

Changes in the rates of initiation/reinitiation and rates of elongation of polypeptide chains

Another possible mechanism of regulation is changing the rates of initiation, reinitiation and/or elongation. This has been described in the regulation of bovine parathyroid chromogranin A (CgA) by $1,25(\text{OH})_2\text{D}_3$ in which a lack of correlation between CgA gene transcription and CgA protein levels were due to a decrease in the rate of peptide elongation without a concomitant decrease in initiation (Mouland & Hendy, 1992). This type of regulatory mechanism has also been described for ornithine aminotransferase synthesis in hepatocytes isolated from rat livers in which a high protein diet resulted in a 100-fold increase in ornithine aminotransferase but only a 2-fold rise in mRNA (Mueckler *et al.* 1983). This discrepancy between the glucagon-induced rise in mRNA levels and the greater increase in enzyme synthesis was shown to be due to an increase in the rates of initiation and elongation, that is, an increase in translational yield. In addition, the polysomal content of ornithine aminotransferase mRNA increased and this was directly proportional to the increase in the initiation rate.

AIMS OF THIS STUDY

The aims of this study were to investigate the post-transcriptional regulatory mechanisms in bovine parathyroid cells in the presence of low calcium. Using differential centrifugation, the distribution of mRNA on

bound polysomes, free polysomes, free mRNA and mRNA bound to ribonucleoproteins were analysed by Northern blotting. Furthermore, total RNA was fractionated using sucrose density gradient ultracentrifugation followed by Northern analysis. This method gives an indication of a change in polysomal size as well as the presence of a cytoplasmic pool of mRNA.

The binding site for the receptor for $1,25(\text{OH})_2\text{D}_3$ in the bovine parathyroid hormone gene was localised using filter binding, Southwestern, and gel mobility shift assays. In order to investigate whether fragments of the promoter region were transcriptionally active, plasmids containing these fragments were linked to the bacterial reporter enzyme gene, chloramphenicol acetyltransferase (CAT) and the effect of $1,25(\text{OH})_2\text{D}_3$ on these constructs was investigated by transient transfection into adherent opossum kidney cells.

Studies of the effects of low calcium on PTH synthesis will be described in chapter 2. This is followed in chapter 3 by a description of the studies of binding of vitamin D_3 receptors to the PTH gene followed by CAT assays in chapter 4. General methods are described in Appendix I, whilst those methods that were optimised or validated for each study are presented in the relevant chapters. Finally, regulation of PTH synthesis will be put in context in the concluding chapter.

CHAPTER 2

REGULATION OF PTH SYNTHESIS BY LOW CALCIUM

INTRODUCTION

The synthesis and secretion of PTH by the parathyroid glands is controlled by a negative feedback mechanism, regulated by the blood level of ionized calcium, and is influenced by $1,25(\text{OH})_2\text{D}_3$. PTH is unlike most secreted proteins, in that a decrease in intracellular ionized calcium elevates PTH concentrations whilst an increase in ionized calcium suppresses PTH in the circulation. Parathyroid cells are thought to sense extracellular calcium through G-protein-coupled calcium receptors (Racke *et al.* 1991) and a calcium receptor clone from bovine parathyroid glands has been characterised (Brown *et al.* 1993) which will help elucidate the mechanism involved in regulating calcium metabolism.

The nucleotide sequence of the bovine PTH gene has been determined using Southern blot hybridisation of genomic DNA which showed that the PTH gene in that clone was contained within a 7000 base pairs (bp) EcoRI fragment. To clone the gene, liver DNA was digested with EcoRI and fragments in the range of 4,000 to 12,000 bp were isolated by sucrose density gradient ultracentrifugation (Weaver *et al.* 1984). The DNA was used to produce a partial library in λ phage Charon 30 and several clones were isolated by plaque filter hybridisation using PTH cDNA probes. Subsequently the gene was sequenced by the method of Maxam & Gilbert and shown to consist of three exons (shaded boxes, figure 2.1) separated by two introns (thin lines). Exon 1 contains 95 bp that codes for the 5'-untranslated region (UTR) except for the 5 bp preceding the initiator codon. Exon 2 contains 91 bp that correspond to the 5 bp from the 5'-UTR to the region that codes for

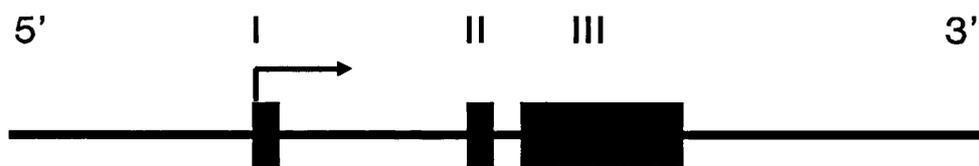


Figure 2.1. The bovine PTH gene consists of three exons I, II, and III, two intervening sequences (introns), the 5' and 3'-flanking regions. \curvearrowright indicates the transcription start site.

the pre-sequence and the first four amino acids of the pro-sequence of preproPTH. Exon 3 contains 486 bp that code for the remainder of the pro-sequence, PTH and the 3'-UTR. These are separated by two introns of 1714 and 119 bp respectively.

The presence of two adjacent TATA boxes about 30 bp apart in the 5'-flanking region of the bovine PTH gene which direct initiation of transcription was demonstrated by S1 nuclease mapping (Weaver et al. 1984). Transcription is initiated less frequently from the more upstream site, although both of these appear to be functional, however, only a single band of preproPTH mRNA is observed by Northern analysis (Brookman et al. 1986). The 5' end of rat PTH mRNA was also analysed by S1 nuclease mapping and transcription was found to be initiated from a single site (Heinrich et al. 1984). Three mammalian genes (human, bovine and rat) resemble each other at the nucleotide level with overall homology of the nucleotide sequences of the bovine and human sequences being about 70%; the rat/bovine is 58% and the rat/human is 61% (Kemper, 1984). Furthermore, the three proteins coded for by the gene also share homology in the amino acid sequences, the human and bovine sequences are identical in 85% of their amino acid residues, whilst the rat protein has diverged but still maintains 75% identity with each of the other two proteins.

Studies of the regulation of PTH synthesis and secretion in vitro have indicated that in short-term incubations (4-7 hours), the effect of high extracellular calcium on bovine parathyroid cells reduced PTH secretion with no detectable change in preproPTH mRNA levels (Heinrich et al. 1983;

Brookman *et al.* 1986), whilst longer incubation periods (16-24 hours), decreased preproPTH mRNA levels in addition to a fall in secretion (Russell *et al.* 1983; Brookman *et al.* 1986). The decrease in secretion may be mediated by high extracellular calcium enhancing the intracellular degradation of PTH in the cell by cathepsin D (Habener *et al.* 1975) and/or inhibiting the release of PTH from storage granules or affecting the release of PTH fragments secreted directly through the membrane. Farrow *et al.* (1988) have shown that the reduction was not due to a decrease in release of PTH from storage granules but due to a significant decrease in PTH synthesis. This was shown using bovine parathyroid cells pulsed with [³⁵S] methionine, in which incorporation of [³⁵S] methionine into intracellular proteins in cells incubated in high calcium was reduced by approximately 50% in 24 hours compared to cells incubated in normal calcium. Furthermore, there was no evidence of degradation of newly synthesised hormone, which would have indicated some regulation by this mechanism. The decrease in mRNA levels seen in the presence of high calcium may be due to decreased transcription and/or increased degradation of mRNA and Sherwood *et al.* (1987) using nuclear run-off assays in bovine parathyroid cultures *in vitro*, have shown that high calcium directly decreased the rate of transcription by 40%. In contrast, in cultured human parathyroid adenomas, *de novo* PTH synthesis continues in the presence of high extracellular calcium despite a fall in preproPTH mRNA levels (Farrow *et al.* 1988). Studies of ³⁵S-methionine incorporation into secreted hormone showed that the continued secretion was not of stored hormone, but of newly-synthesised hormone, suggesting that translation in

adenomas is not affected by high calcium.

Studies of bovine parathyroid cells cultured in low calcium have shown an increase in PTH secretion with no change in steady-state preproPTH mRNA levels (Brookman *et al.* 1986). In addition, Sherwood *et al.* (1987) have shown that low calcium did not increase PTH gene transcription in normal bovine glands *in vitro* after 24 hours. The lack of correlation between changes in mRNA levels and PTH synthesis suggests the existence of a post-transcriptional site of regulation in addition to that of gene transcription. This is in contrast to *in vivo* results which have demonstrated an increase in steady-state mRNA levels (Yamamoto *et al.* 1989; Naveh-Manly & Silver, 1990). Using a dietary model in rats, the latter group have shown that calcium and 1,25(OH)₂D₃-deficient rats had a 10-fold increase in steady-state mRNA levels. However, neither group correlated mRNA levels with secretion in order to determine whether there was a parallel increase in PTH synthesis and in addition, whether this increase in PTH mRNA levels was due to changes in gene transcription rates or mRNA half-life. Nuclear transcription run-off experiments and studies of stability could resolve this question. The exact mechanisms causing these differences between *in vivo* and *in vitro* results are not apparent. It may be that the basal levels of calcium and 1,25(OH)₂D₃ *in vivo* are different to that *in vitro* and in addition are more easily controlled *in vitro*.

AIMS OF THE STUDY

The aims of the study presented here were to investigate post-transcriptional regulatory mechanisms in bovine parathyroid cells in the presence of 0.4 and 1.0 mM calcium. In this study, 0.4 mM calcium is referred to as low and 1.0 mM as normal extracellular calcium levels. The effect of calcium on steady-state preproPTH mRNA levels was investigated using dot-blot assays in which cytosolic mRNA was hybridised to cDNA probes. This assay is widely used and has the advantage of allowing quantification of mRNA in multiple samples. The various pools in which cytoplasmic mRNA may exist in a cell namely, that actively being translated by membrane-bound or free polysomes, that which may not be active, that is, complexed to ribonucleoproteins (RNP) or present as free mRNA were investigated using differential ultracentrifugation and analysed by Northern blotting. The possibility that the increase in PTH synthesis could be due to recruitment of preproPTH mRNA present in the cytosol possibly bound to RNPs or other proteins was also investigated by fractionation of total RNA from bovine parathyroid glands incubated in 0.4 or 1.0 mM calcium on sucrose density gradients. This method separates active polysomes from non-translating monosomes and ribosomal subunits from free mRNPs and free mRNA. In addition this method gives an indication of changes in polysomal size.

In this chapter, I will present the methods used and results of the above studies and discuss the putative control mechanisms with respect to post-transcriptional regulation of PTH synthesis.

METHODS

Cell culture of parathyroid tissue

Bovine parathyroid glands were transported on ice from the abattoir in Waymouth's medium containing 20 mM Hepes (pH 7.3) and 0.06% (v/v) sodium bicarbonate to the laboratory. Glands were washed briefly in 70% ethanol, rinsed in fresh medium, trimmed and minced finely prior to digestion. Sterile conditions were used throughout. The minced tissue was centrifuged briefly and resuspended in Waymouth's medium containing 20 mM Hepes (pH 7.3), 0.06% (w/v) sodium bicarbonate, and digested with 2.5 mg/ml collagenase and 40 μ g/ml DNase for 90 minutes at 37°C. During digestion, cells were disrupted by pipetting. Cells were washed three times in phosphate-buffered saline (PBS) and resuspended in Waymouth's medium supplemented with 10% (v/v) foetal calf serum, penicillin (100 Units/ml), streptomycin (100 μ g/ml) and mycostatin (25 Units/ml). Aliquots of 1.5 ml cell suspension were distributed into 35 mm petri dishes in duplicate for cytosolic RNA and in 15 ml aliquots in 90 mm petri dishes for polysomal studies and fractionation of total RNA. Plates were incubated at 37°C in 5% CO₂ and after 2 days preincubation, medium was replaced with that containing 0.4 and 1.0 mM calcium or with each of these calcium concentrations containing 5 μ g/ml actinomycin D for the polysomal study. In the cytosolic RNA study, medium was replaced with that containing 0.4 and 1.0 mM calcium with 2.5, 5.0 and 10.0 μ g/ml actinomycin D where appropriate. During the incubation period, medium was replaced every 24 hours and cells were incubated in these conditions for 24 or 48 hours after which time they

were harvested.

Preparation of RNA

Solutions used in the preparation of RNA were from autoclaved stocks and all glassware and plasticware were treated with 0.1% diethyl pyrocarbonate (DEPC) in distilled water and autoclaved before use.

Cytosolic mRNA

Cells were harvested, transferred into sterile microfuge tubes and pelleted at 10 000 g at 4°C for 15 minutes. Cell pellets were homogenised in 50 μ l 10 mM Tris-HCl (pH 7.4) with 5% Nonidet P-40 (NP40) (5 μ l) using sterile miniglass pestles (Jencons) and centrifuged at 10 000g for 10 minutes at 4°C. The resulting supernatant (25 μ l) was denatured with 15 μ l 20X SSC and 10 μ l 37% formaldehyde (w/v) at 60°C for 15 minutes. Serial dilutions of 1:5, 1:10, 1:20 and 1:40 were made in 20 X SSC before being blotted onto 0.45 μ m nitrocellulose filters pre-soaked in 20 X SSC using a filtration manifold (Schleicher & Schuell). The filters were allowed to dry at room temperature before being baked at 80°C for 90 minutes.

Preparation of polysomal RNA

Differential centrifugation was used to separate the different pools in which preproPTH mRNA may exist in the cell. Preparation of polysomal RNA was optimised using cultured bovine parathyroid cells incubated in 1.0 mM calcium. Cells were homogenised in 50 mM Tris-HCl (pH 7.4) buffer

containing 0.25 M sucrose, 75 mM KCl, 1 mM DTT, 1 μ g/ml cycloheximide and 0.5 mg/ml heparin. The homogenates were centrifuged at 7 500 g for 30 minutes in a MSE 18 centrifuge and the resulting post-mitochondrial supernatant was centrifuged at 100000 g for 12 minutes initially in a Beckman near vertical rotor (NVT 65), subsequently the centrifugation time was increased to 90 minutes. All procedures were performed at 4°C. After centrifugation, the pellets which contain the membrane-bound polysomes were resuspended in 5 ml 50 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose, 50 mM MgCl₂, 250 mM KCl, 1 μ g/ml cycloheximide, 1 mM DTT, 2 mg/ml heparin, 0.1% sodium deoxycholate and 0.1% Triton X-100. In some experiments, MgCl₂ was replaced by 20 mM EDTA. The resuspended pellets were left at 4°C for 15 minutes to extract membrane-bound polysomes. The resuspended pellets and supernatants containing the free polysomes were layered onto discontinuous sucrose gradients of 1.3 M and 2.0 M sucrose and centrifugation was performed at 180 000g for 20 hours at 4°C in a Beckman NVT65 rotor. Further centrifugation at 245 000 g for 2.5 hours at 4°C resulted in a pellet which contains the ribonucleoproteins (RNPs) and the supernatant containing the free mRNA. The resulting pellets were resuspended in 20 mM Tris-HCl (pH 7.4) containing 0.2 M NaCl, 40 mM EDTA and 1% SDS at 100°C for 5 minutes, cooled to 30°C and incubated with 0.5 mg/ml proteinase K at 30°C for 10 minutes. Samples were adjusted to 0.1M Tris-HCl (pH 9.0) and SDS to 1% (final concentration) was added and extracted twice with phenol:chloroform. RNA was alcohol precipitated, recovered by centrifugation and resuspended in DEPC-water. Purity of the

preparation was determined by measuring the optical density at 260 nm and 280 nm. Typically, ratios of approximately 1.7-1.9 were obtained. Absorbance at 260 nm was used to calculate the RNA concentration (A_{260} of 1.0 = 40 $\mu\text{g/ml}$).

The microsomal enzyme, glucose-6-phosphatase was used as a marker enzyme and initially in the optimisation of this method, only 10% of the glucose-6-phosphatase activity was detected in the microsomal fraction, with the remainder in the free and mRNP fractions. Thus, subsequently homogenates were centrifuged at 100 000 g for 90 minutes at 4°C in order to pellet the membrane-bound polysomes. Northern blot analysis of these fractions revealed very little mRNA in the membrane-bound polysomal fraction (figure 2.2) with most being present in the free fraction as well as complexed to mRNPs. Whilst the free polysome fraction probably contained some contaminating small vesicles of the endoplasmic reticulum, it is unlikely to have contained any monosomal matter, as this does not sediment through 2.0 M sucrose (Wettstein *et al.* 1963). In addition, any RNP species would have remained in the post-polysomal supernatant and thus would not have been measured in this study.

The effect of the chelating agent EDTA on preproPTH mRNA associated with membrane-bound polysomes was investigated. In the presence of EDTA, mRNA on membrane-bound polysomes were reduced markedly (figure 2.3) in cells incubated in 1.0 mM calcium, indicating that membrane-bound polysomes were dissociated resulting in ribosomal subunits.

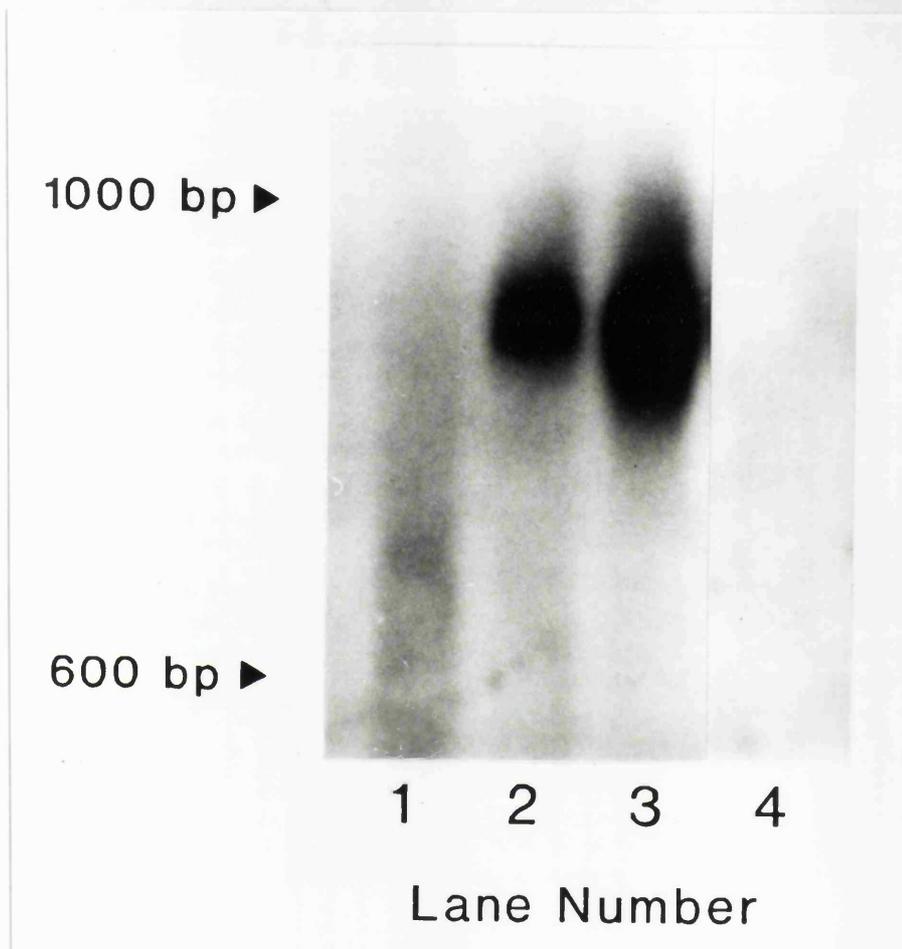


Figure 2.2. Autoradiograph of Northern blotting following differential centrifugation showing preproPTH mRNA associated with membrane-bound (lane 1) and free polysomes (lane 2), ribonucleoproteins (RNP, lane 3) and free mRNA (lane 4) in bovine parathyroid cells incubated in 1.0 mM calcium for 48 hours. The position of molecular size markers are shown on the left.

Isolation of total RNA by sucrose density gradient ultracentrifugation

Parathyroid cells incubated in 0.3 mM and 1.0 mM calcium for 48 hrs were homogenised in 50 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose, 75 mM KCl, 1 mM DTT, 10 mM MgCl₂, 10 µg/ml cycloheximide, 0.5 mM heparin and 20 Units RNase inhibitor (Boehringer Mannheim). In some experiments, MgCl₂ was replaced by 20 mM EDTA. Homogenates



Glucose-6-phosphatase assay

Extracts (50 µl) were incubated in 20 mM sodium acetate solution (pH 4.6)

Figure 2.3. Autoradiograph following Northern blotting of preproPTH mRNA associated with membrane-bound polysomes in bovine parathyroid cells incubated in 1.0 mM calcium in the presence of either MgCl₂ (lanes 1 and 2) or EDTA (lanes 3 and 4).

Fractionation of total RNA by sucrose density gradient ultracentrifugation

Parathyroid cells incubated in 0.4 mM and 1.0 mM calcium for 48 hrs were homogenised in 50mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose, 75 mM KCl, 1 mM DTT, 10 mM MgCl₂, 10 µg/ml cycloheximide, 0.5 mM heparin and 20 Units RNase inhibitor (Boehringer Mannheim). In some experiments, MgCl₂ was replaced by 20 mM EDTA. Homogenates were centrifuged at 2800 g for 10 minutes at 4°C in a MSE 18 centrifuge. Sodium deoxycholate (0.1%, w/v) and Triton X-100 (0.1%, w/v) were added to the homogenates, left on ice for 15 mins and layered onto linear gradients consisting of 10-50% sucrose in homogenisation buffer without 0.25 M sucrose. The gradients were centrifuged at 85000g in a Beckman swing-out rotor (SW 41) for 4 hrs at 4°C. Typically, 28 X 0.5 ml fractions were collected from the bottom of the tube at 4°C and RNA precipitated by adding 3 M sodium acetate and absolute alcohol at -70°C. RNA was extracted twice with phenol:chloroform, recovered by centrifugation and resuspended in 12 µl DEPC-water. This was divided for Northern analysis and staining for ribosomal RNA with ethidium bromide and dried under vacuum. Samples were resuspended in 1 µl DEPC-water and denatured in glyoxal as described below.

Glucose-6-phosphatase assay

Extracts (50 µl) were incubated in 20 mM sodium acetate solution (pH 4.6, 400 µl) containing 20 mM glucose-6-phosphate for 20 minutes at 37°C and the reaction was terminated by the addition of 2 volumes of 10% (w/v)

trichloroacetic acid. After centrifugation at 1500 g for 15 minutes, the supernatant was incubated with ascorbic acid (1%) and ammonium molybdate (0.25%) at 45°C for 20 minutes. The absorbance was measured at 520 nm.

Northern blot analysis

Polysomal RNA samples (20 μg) and 50 μg RNA molecular marker III (Boehringer Mannheim) were denatured with a mixture of dimethyl sulphoxide (9 M), glyoxal (2 M) and 0.013 M NaHPO_4 (pH 7.0) containing tRNA (6 $\mu\text{g}/\mu\text{l}$) at 50°C for 60 minutes. After cooling on ice, 2.5 μl glycerol was added and samples were separated on agarose gels (0.1%) in 0.01 M NaHPO_4 (pH 7.0) buffer at 90 V. During electrophoresis, the buffer was recirculated using a peristaltic pump to maintain the pH since glyoxal dissociates from RNA at pH > 8.0. After electrophoresis, RNA was transferred by capillary blotting to 0.45 μm nitrocellulose filters in 20 X SSC at 4°C for 16 hours. Filters were baked at 80°C for 1.5 hours before being used for Northern analysis.

Dot-blots and Northern blots were prehybridised and hybridised according to the method of Thomas (1980) to a cDNA probe covering the entire coding region for the bovine preproPTH gene (pPTHm29) (Kronenberg *et al.* 1979). Nitrocellulose filters were prehybridised in 0.05 M NaH_2PO_4 buffer containing 5 X SSC, 50% deionised formamide, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone (PVP), 0.01% (w/v) BSA and 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA at 42°C for 16 hours and hybridised in the above buffer containing 10% dextran sulphate and 10^6 cpm/ml denatured cDNA probe

labelled with ^{32}P - α -dCTP (3.7 MBq) as described in Appendix I at 42°C for 16 hours. Filters were washed 4 times in 2 X SSC and 0.1% SDS at 20°C for 15 minutes followed by 4 times in 0.1 X SSC and 0.1% SDS at 50°C for 15 minutes. The filters were exposed to X-ray film at -70°C with intensifying screens. After autoradiography, RNA was quantified by densitometry at 525 nm. All results were expressed as means \pm S.D. Statistical significance was determined using the Wilcoxon test with $p < 0.05$ as the lowest level of significance. Results of dot-blot assays were expressed relative to cytosolic proteins concentrations determined by the method of Lowry *et al.* (1951) as described in Appendix I.

Filters probed with cDNA to PTH were stripped by washing in 5 mM Tris-HCl (pH 8.0) buffer containing 0.2 mM EDTA, 0.05% sodium pyrophosphate, 0.02% (w/v) BSA, 0.02% (w/v) Ficoll and 0.02% (w/v) PVP for 2 hrs at 62°C and re-probed with β -actin labelled by nick translation as described in Appendix I. Filters were prehybridised, hybridised and autoradiographed as described above.

Staining for ribosomal RNA

RNA fractions from sucrose gradients were denatured in glyoxal, electrophoresed through agarose gels (1%) in 0.01 M NaHPO_4 buffer (pH 7.0) and stained with ethidium bromide (1 $\mu\text{g}/\text{ml}$) for 30 mins at 20°C to identify the distribution of the ribosomes and their subunits. Ribosomal bands were visualised under UV light.

RESULTS

Validation of dot-blot assays

This technique was validated using cytoplasmic mRNA prepared from bovine parathyroid cells incubated in 1.0 mM calcium. Figure 2.4 (A) shows an example of an autoradiograph after serial dilutions of denatured cytosol were hybridised to radiolabelled preproPTH cDNA in quadruplicate. This was scanned on a densitometer and plotted (figure 2.4 B). A linear relationship between the amount of cytoplasmic extract and the intensity of signal after hybridisation was observed between 1/5 and 1/40 dilutions. The intensity of the 1/10 and 1/20 dilution points (in the linear part of the curve as indicated by the arrows) were used to determine the mRNA levels in each sample. Proteins were measured by the Lowry *et al.* (1951) method and cytosolic mRNA was expressed as densitometer readings/mg protein.

Effect of low calcium on steady-state preproPTH mRNA levels

To confirm that incubation in calcium does not alter steady-state preproPTH mRNA levels, cells were incubated in 0.4 and 1.0 mM calcium for 24 or 48 hours and cytoplasmic mRNA prepared. Quantitative analysis of dot-blot hybridisation after serial dilutions of denatured cytosol revealed that incubation in 0.4 mM calcium did not increase cytoplasmic steady-state preproPTH mRNA levels relative to those in cells incubated in 1.0 mM calcium ($100 \pm 15\%$ and $100 \pm 14\%$ of control, respectively) after 24 hours (figure 2.5). Similarly, there was no significant difference after 48 hours.

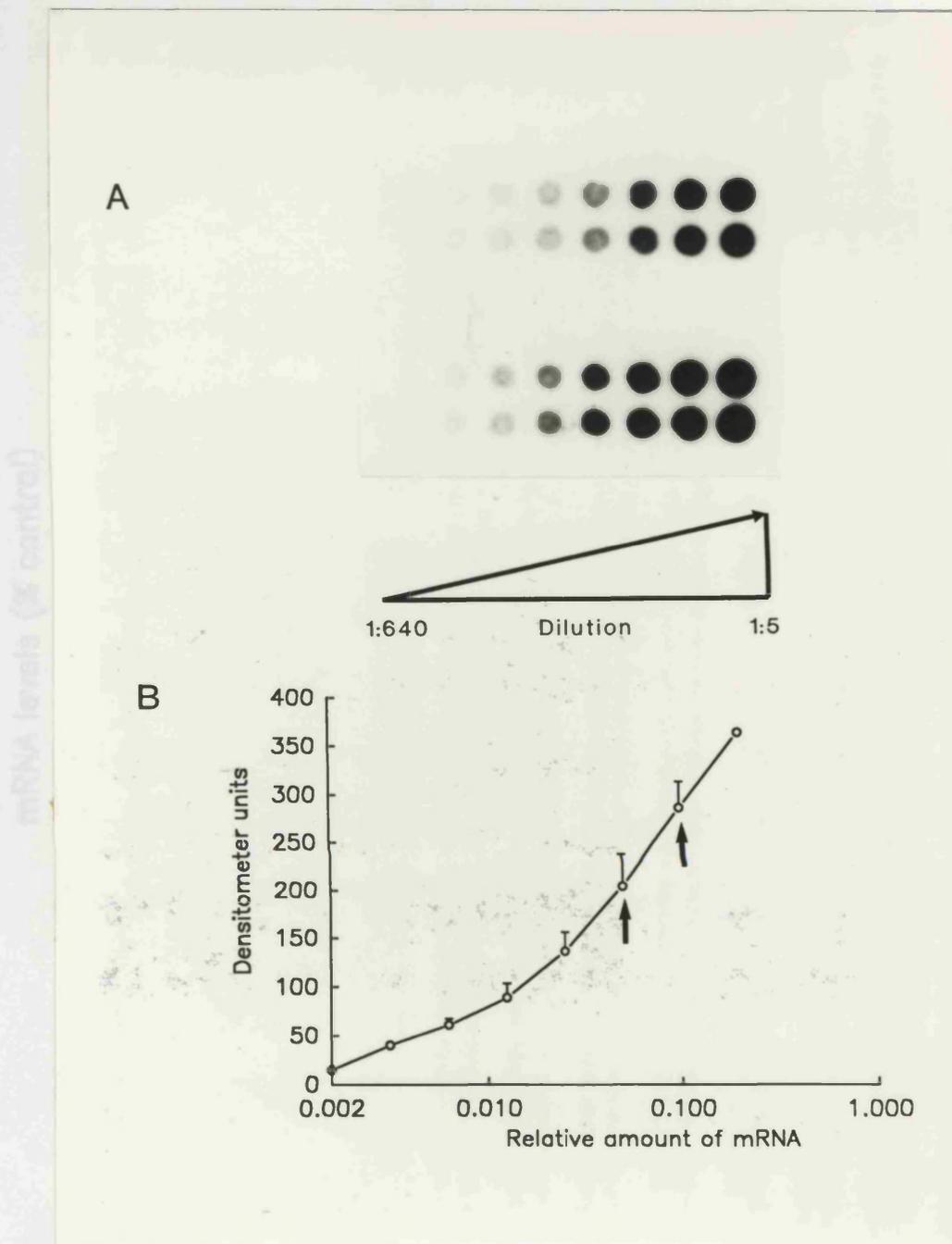


Figure 2.4 (A) Autoradiograph of a dot-blot of serial dilution (1/5 to 1/640) of cytoplasmic mRNA prepared from cells incubated in 1.0 mM calcium for 24 hours in quadruplicate. The triangle indicates increasing amounts of cytosol used for dilution.

(B) Densitometric scanning of autoradiograph showing the relationship between the amount of preproPTH mRNA and the intensity of the dot-blot. The intensity of the 1/10 and 1/20 dilution points were used to determine mRNA levels in each sample (indicated by arrows).

Effect of calcium and actinomycin D on the stability of steady-state preproPTH mRNA

In order to assess whether calcium has an effect on the stability of steady-state mRNA levels, cells were incubated in medium containing 0.4 or 1.0 mM calcium in the presence and absence of actinomycin D. Cells were harvested at 24 and 48 hours and cytosolic RNA was quantitated by dot blot assays.

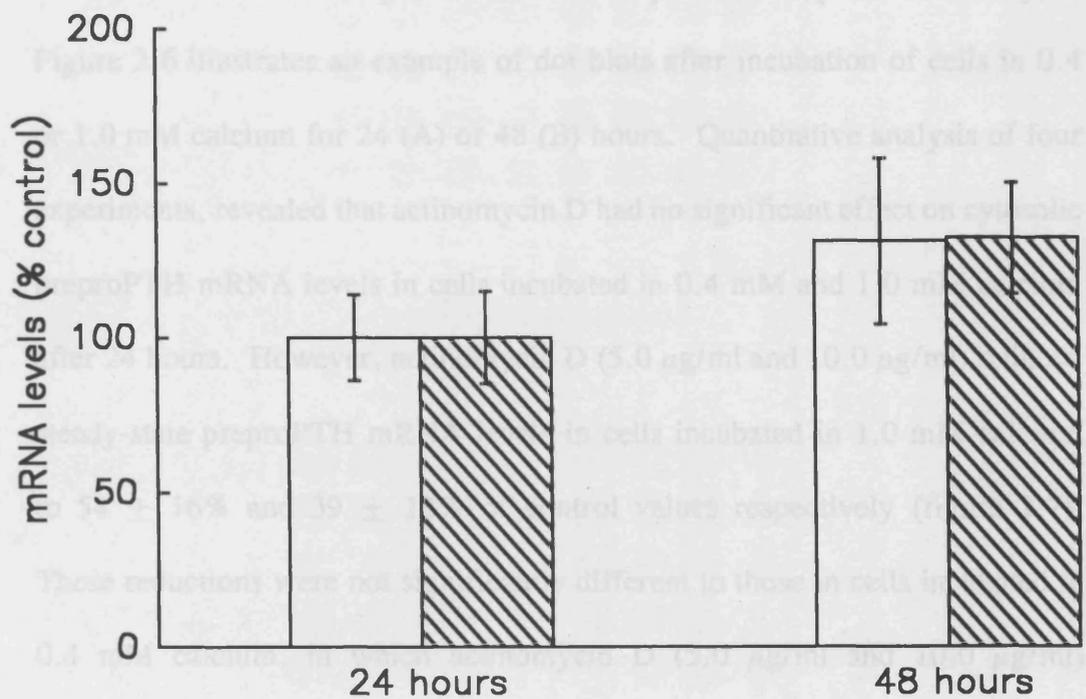


Figure 2.5. Quantitative analysis of steady-state preproPTH mRNA measured by dot-blot assays of parathyroid cells incubated in 0.4 (▨) or 1.0 (□) mM calcium for 24 or 48 hours. Results are expressed relative to control values (incubation in 1.0 mM calcium) for 24 hours and shown as mean \pm SD., n=3.

Effect of calcium and actinomycin D on the stability of steady-state preproPTH mRNA

In order to assess whether calcium has an effect on the stability of steady-state mRNA levels, cells were incubated in medium containing 0.4 or 1.0 mM calcium in the presence and absence of actinomycin D. Cells were harvested at 24 and 48 hours and cytosolic RNA was quantitated by dot blot analysis. Figure 2.6 illustrates an example of dot blots after incubation of cells in 0.4 or 1.0 mM calcium for 24 (A) or 48 (B) hours. Quantitative analysis of four experiments, revealed that actinomycin D had no significant effect on cytosolic preproPTH mRNA levels in cells incubated in 0.4 mM and 1.0 mM calcium after 24 hours. However, actinomycin D (5.0 $\mu\text{g/ml}$ and 10.0 $\mu\text{g/ml}$) reduced steady-state preproPTH mRNA levels in cells incubated in 1.0 mM calcium to $54 \pm 16\%$ and $39 \pm 12\%$ of control values respectively (figure 2.7). These reductions were not significantly different to those in cells incubated in 0.4 mM calcium, in which actinomycin D (5.0 $\mu\text{g/ml}$ and 10.0 $\mu\text{g/ml}$) reduced preproPTH mRNA levels to $57 \pm 13\%$ and $45 \pm 5\%$ of control values respectively. Actinomycin D (2.5 $\mu\text{g/ml}$) reduced steady-state preproPTH mRNA levels in cells cultured in 0.4 mM calcium to $35 \pm 5\%$ and in 1.0 mM calcium to $77 \pm 3\%$ of control values after 48 hours. Thus, actinomycin D (5 $\mu\text{g/ml}$) reduced steady-state mRNA levels in cells incubated in both 0.4 and 1.0 mM calcium to about 50% of control after 48 hours, suggesting similar half-lives in both conditions.

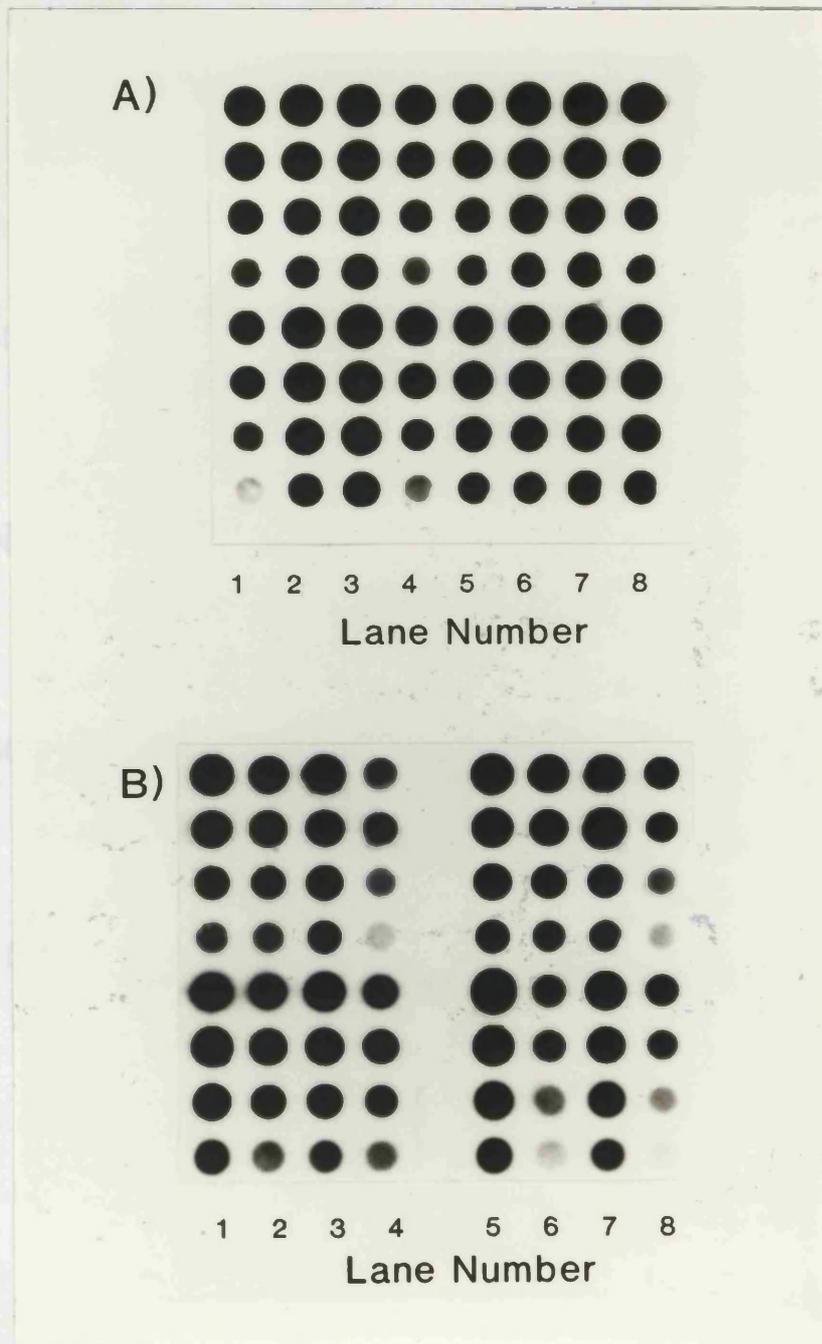


Figure 2.6. (A) Autoradiograph of dot blot assays of cytosolic RNA prepared from cells incubated in 0.4 or 1.0 mM calcium (lanes 1 and 5, respectively) or with the addition of actinomycin D (2.5, 5.0 and 10.0 $\mu\text{g}/\text{ml}$) (lanes 2-4) after 24 hour or (B) after 48 hours. Reading vertically are four dilutions of duplicate plates.

Distribution of preproPTH mRNA

Differential centrifugation was used to separate some of the cytoplasmic pools in which preproPTH mRNA may be localized within cells. Northern blot analysis of RNA extracted from membrane-bound polyosomes and mRNA of cells incubated in 0.4 mM, 1.0 mM and 3.0 mM calcium for 48 hours revealed that preproPTH mRNA was detectable only on membrane-bound polyosomes (figure 2.8). In addition, there was no non-ribosomal

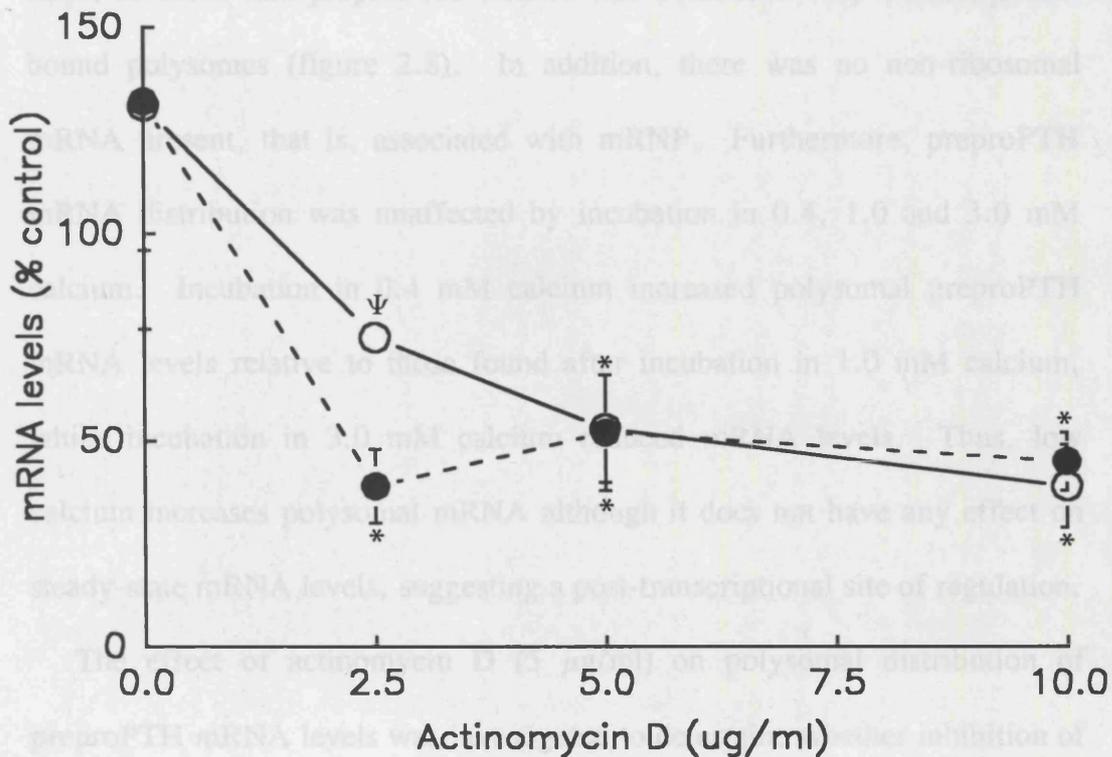


Figure 2.7 shows the effects of actinomycin D on steady-state preproPTH mRNA levels in cells incubated in 0.4 (●) or 1.0 (○) mM calcium for 48 hours. Results are expressed relative to control values (incubation for 24 hours) and shown as mean \pm S.D., $n=4$. * $p < 0.05$ for comparison to control values, $\Psi p < 0.05$ for comparison of cells incubated in 0.4 and 1.0 mM calcium.

Distribution of preproPTH mRNA

Differential centrifugation was used to separate some of the cytoplasmic pools in which preproPTH mRNA may be localised within cells. Northern blot analysis of RNA extracted from membrane-bound and free polysomes and mRNP of cells incubated in 0.4 mM, 1.0 mM and 3.0 mM calcium for 48 hours revealed that preproPTH mRNA was detectable only on membrane-bound polysomes (figure 2.8). In addition, there was no non-ribosomal mRNA present, that is, associated with mRNP. Furthermore, preproPTH mRNA distribution was unaffected by incubation in 0.4, 1.0 and 3.0 mM calcium. Incubation in 0.4 mM calcium increased polysomal preproPTH mRNA levels relative to those found after incubation in 1.0 mM calcium, whilst incubation in 3.0 mM calcium reduced mRNA levels. Thus, low calcium increases polysomal mRNA although it does not have any effect on steady-state mRNA levels, suggesting a post-transcriptional site of regulation.

The effect of actinomycin D (5 $\mu\text{g/ml}$) on polysomal distribution of preproPTH mRNA levels was investigated to determine whether inhibition of transcription by actinomycin D prevented the increase in mRNA levels in the presence of 0.4 mM calcium. Figure 2.9 shows a Northern blot of membrane-bound polysomal preproPTH mRNA prepared from cells incubated in 0.4 mM or 1.0 mM calcium and in the presence and absence of 5.0 $\mu\text{g/ml}$ actinomycin D. As described above, there was an increase in preproPTH mRNA associated with membrane-bound polysomes in the presence of 0.4 mM calcium after 48 hours compared to cells incubated in 1.0 mM calcium.

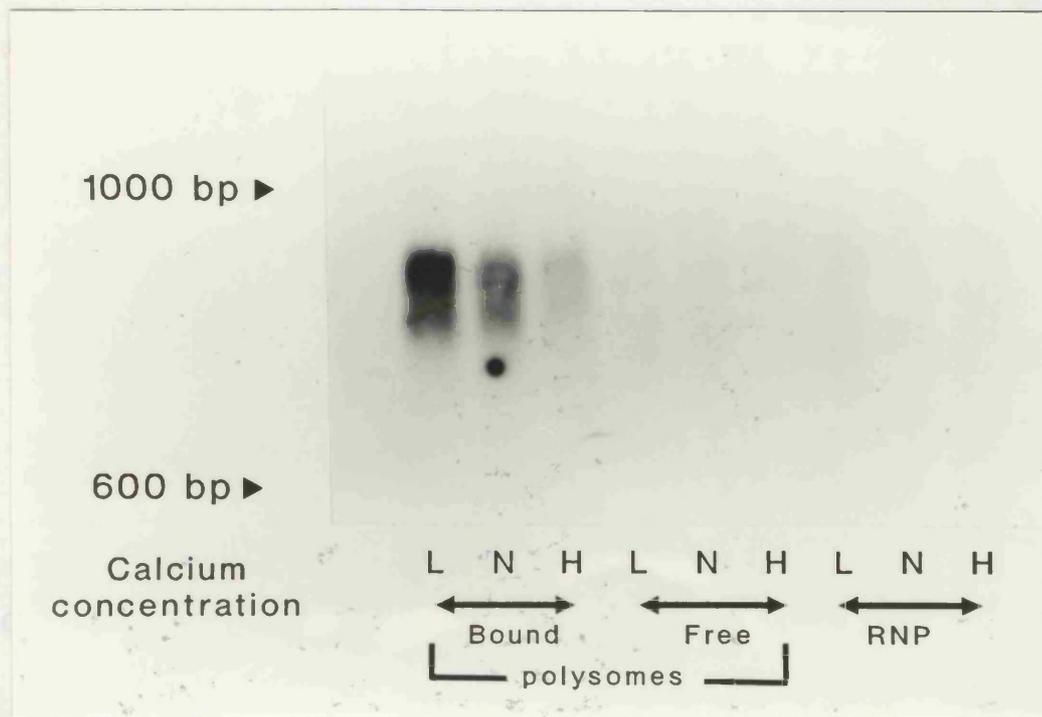
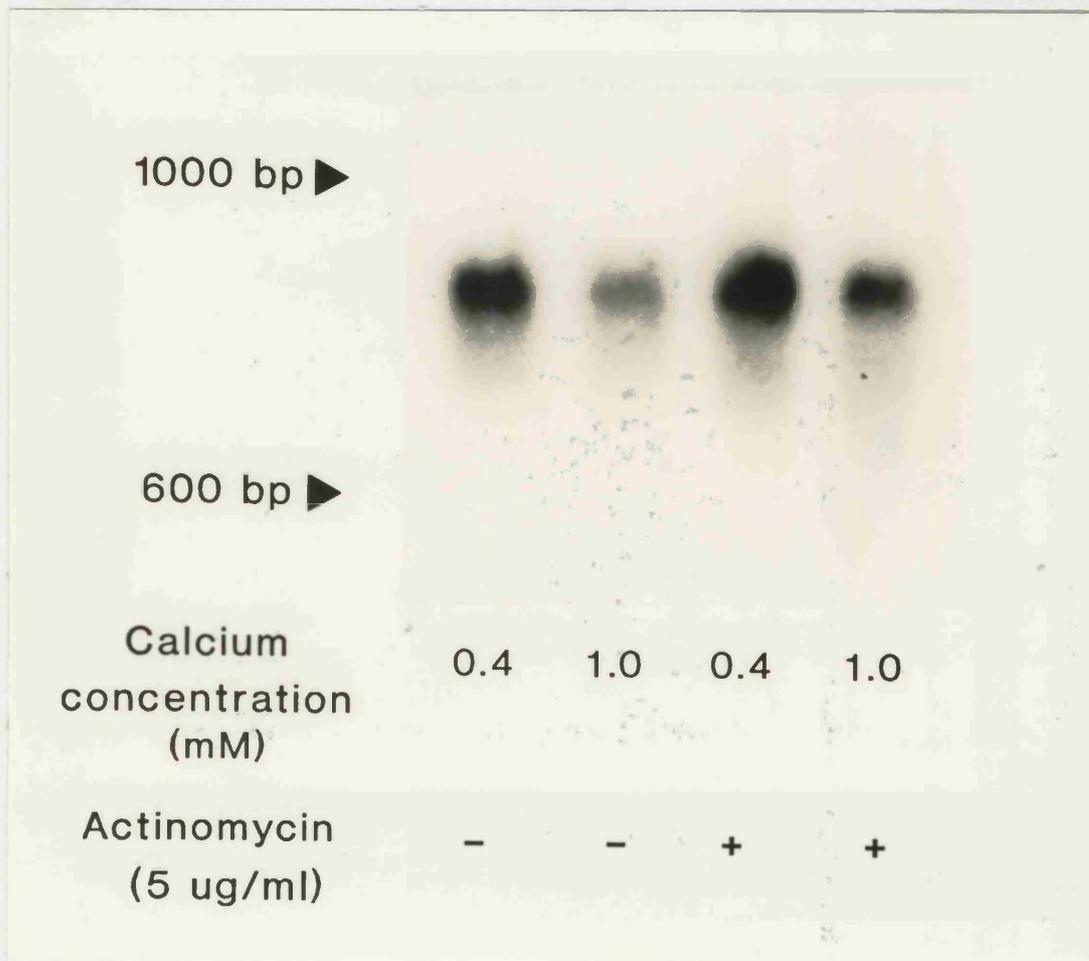


Figure 2.8. Autoradiograph of Northern blotting of preproPTH mRNA

associated with membrane-bound and free polysomes and ribonucleoproteins (RNP) in bovine parathyroid cells incubated in 0.4, 1.0 and 3.0 mM calcium for 48 hours. The position of molecular size markers are shown on the left.

This increase was not abolished in the presence of Actinomycin D. Quantitative analysis of a pool of Northern blots shows in Figure 2.10. Calcium (0.4 mM) increased polyosomal mRNA levels to $200 \pm 16\%$, whereas there was no change in cells incubated in 1.0 mM calcium after 48 hours, that is, $100 \pm 1\%$ of control values. Actinomycin D did not abolish this effect



the polyosomal and ribosomal fractions (1-30, figure 2.11-A). In homogenates to which EDTA (20 mM) had been added prior to layering onto sucrose density gradients, preproPTH RNA was recovered in fractions 15-30 (figure 2.11-B). This illustrates that the polyosomes present in the heavier fraction (1-

Figure 2.9. Autoradiograph of Northern analysis of preproPTH mRNA associated with membrane-bound polysomes in bovine parathyroid cells incubated in 0.4 or 1.0 mM calcium in the absence or presence of actinomycin D (5 $\mu\text{g}/\text{ml}$) for 48 hours. Positions of molecular size markers are indicated on the left.

This increase was not abolished in the presence of actinomycin D. Quantitative analysis of a pool of Northern blots is shown in figure 2.10. Calcium (0.4 mM) increased polysomal mRNA levels to $200 \pm 16\%$, whereas there was no change in cells incubated in 1.0 mM calcium after 48 hours, that is, $100 \pm 1\%$ of control values. Actinomycin D did not abolish this effect despite reducing steady-state preproPTH mRNA levels to about 50% of control (figure 2.7) but increased the levels of polysomal preproPTH mRNA in cells incubated in 1.0 mM calcium to $164 \pm 13\%$ of control values and $331 \pm 43\%$ of control values in cells incubated in 0.4 mM calcium, that is, increases of about 1.6-fold in both cases.

Fractionation of total RNA

To investigate if there was a pool of PTH mRNA not associated with ribosomal RNA, total RNA was fractionated by sucrose gradient ultracentrifugation and analysed by Northern blotting. To validate this method, parathyroid cells were incubated in 1.0 mM calcium in the absence (A) or presence (B) of EDTA (20 mM) for 48 hours (figure 2.11). In cells incubated in 1.0 mM calcium (A), preproPTH RNA was predominantly associated with the polysomal and ribosomal fractions (1-20, figure 2.11 A). In homogenates to which EDTA (20 mM) had been added prior to layering onto sucrose density gradients, preproPTH RNA was recovered in fractions 15-30 (figure 2.11 B). This illustrates that the polysomes present in the heavier fraction (1-20) as seen in figure 2.11 A, dissociated in the presence of EDTA resulting in monosomes and free ribosomal subunits.

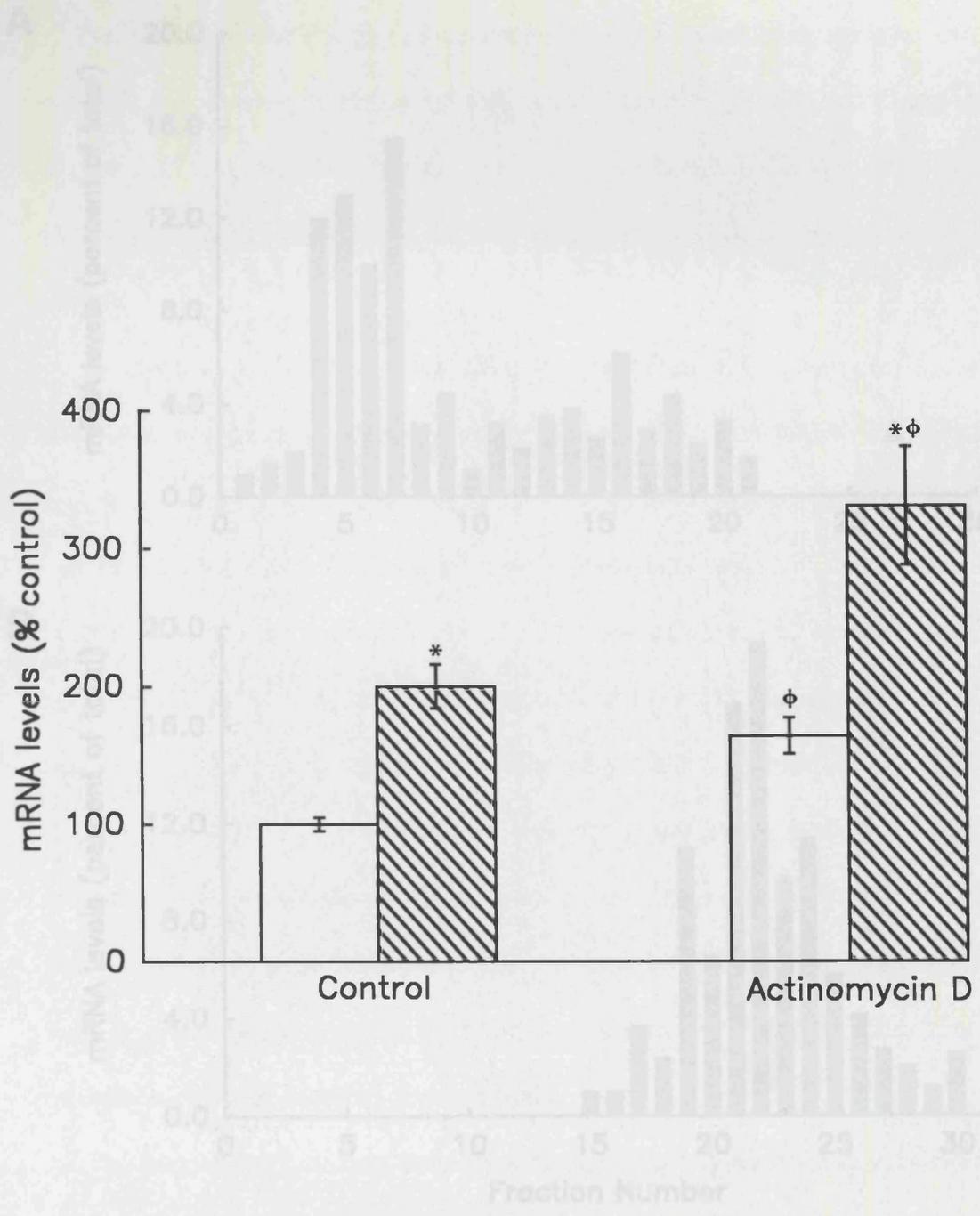


Figure 2.10. Quantitative analysis of Northern blot of preproPTH mRNA associated with membrane-bound polysomes in bovine parathyroid cells incubated in 0.4 (▨) or 1.0 (□) in the absence or presence of actinomycin D (5 μg/ml) for 48 hours. Results are expressed as means ± S.D. (n=2 or 3). * p < 0.05 for comparison of results in 0.4 and 1.0 mM calcium, φ p < 0.05 for comparison of the effects of actinomycin D versus respective controls.

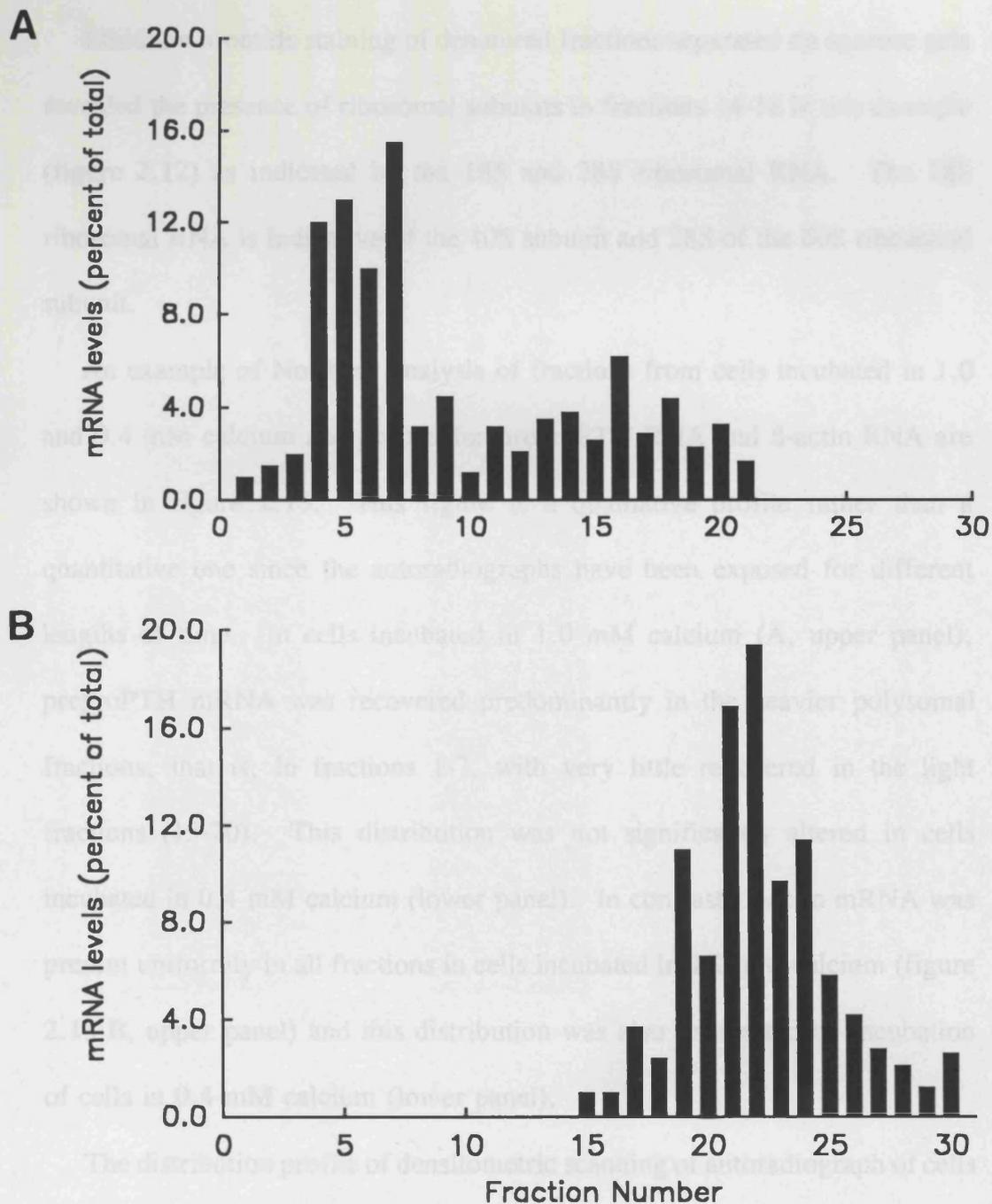


Figure 2.11. (A) Distribution profile of densitometric scanning of Northern blots of total RNA from bovine parathyroid cells incubated in 1.0 mM calcium for 48 hours after fractionation using sucrose gradient ultracentrifugation. PreproPTH RNA was predominantly associated with the polysomal and ribosomal fractions (1-20). (B) illustrates the dissociation of preproPTH RNA from the heavier fractions shown above to fractions 15-30 in the presence of EDTA (20 mM) added prior to layering onto sucrose density gradients.

Ethidium bromide staining of denatured fractions separated on agarose gels revealed the presence of ribosomal subunits in fractions 14-18 in this example (figure 2.12) as indicated by the 18S and 28S ribosomal RNA. The 18S ribosomal RNA is indicative of the 40S subunit and 28S of the 60S ribosomal subunit.

An example of Northern analysis of fractions from cells incubated in 1.0 and 0.4 mM calcium and probed for preproPTH RNA and β -actin RNA are shown in figure 2.13. This figure is a qualitative profile rather than a quantitative one since the autoradiographs have been exposed for different lengths of time. In cells incubated in 1.0 mM calcium (A, upper panel), preproPTH mRNA was recovered predominantly in the heavier polysomal fractions, that is, in fractions 1-7, with very little recovered in the light fractions (15-20). This distribution was not significantly altered in cells incubated in 0.4 mM calcium (lower panel). In contrast, β -actin mRNA was present uniformly in all fractions in cells incubated in 1.0 mM calcium (figure 2.13 B, upper panel) and this distribution was also unaffected by incubation of cells in 0.4 mM calcium (lower panel).

The distribution profile of densitometric scanning of autoradiograph of cells incubated in 1.0 mM calcium and 0.4 mM calcium are shown in figure 2.14 A and B. Ribosomal RNA as revealed by ethidium bromide, was recovered in fractions 16-19 and 15-18 in cells incubated in 1.0 and 0.4 mM calcium respectively, and is indicated by the solid line (figure 2.14). RNA recovered was designated into three groups; (a) polysomal containing fractions preceding those in which ribosomal RNA was observed, (b) ribosomal, and (c) those in

A

PreproPTH RNA

1.0 mM
Calcium2.5 mM
Calcium

Fraction number

1

12

←28S

←18S

B

β-ACTIN RNA

1.0 mM
Calcium0.4 mM
Calcium

Fraction number

13

24

←28S

←18S

Figure 2.12. Ethidium bromide staining of sucrose density gradient fractions from total RNA. The positions of the 18S and 28S ribosomal subunit RNAs are marked.

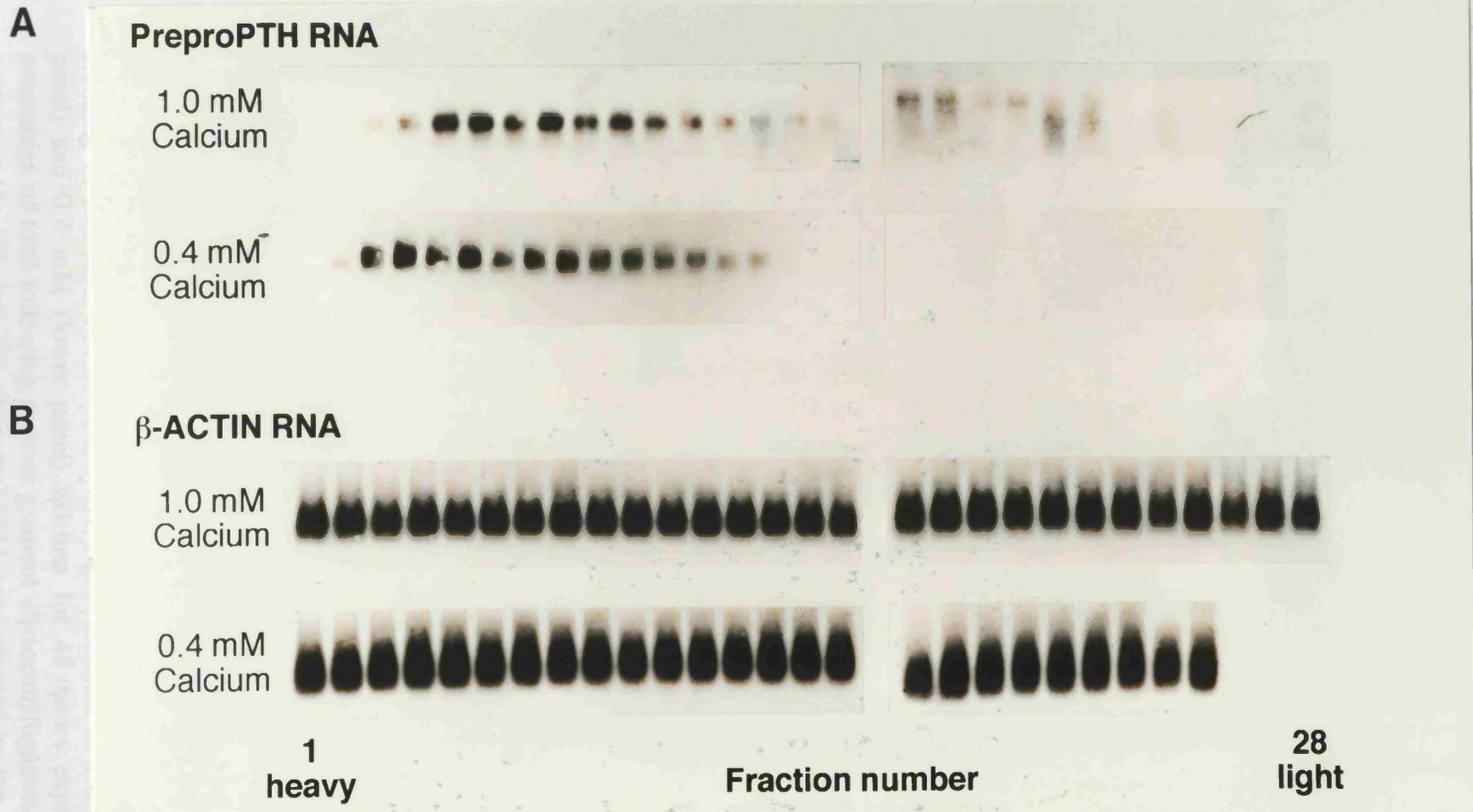


Figure 2.13 Autoradiograph of Northern analysis of total RNA from bovine parathyroid cells incubated in 1.0 mM calcium (A) and 0.4 mM calcium (B) for 48 hours after fractionation using sucrose gradient ultracentrifugation (upper panel) and equivalent filters probed with β -actin (lower panel).

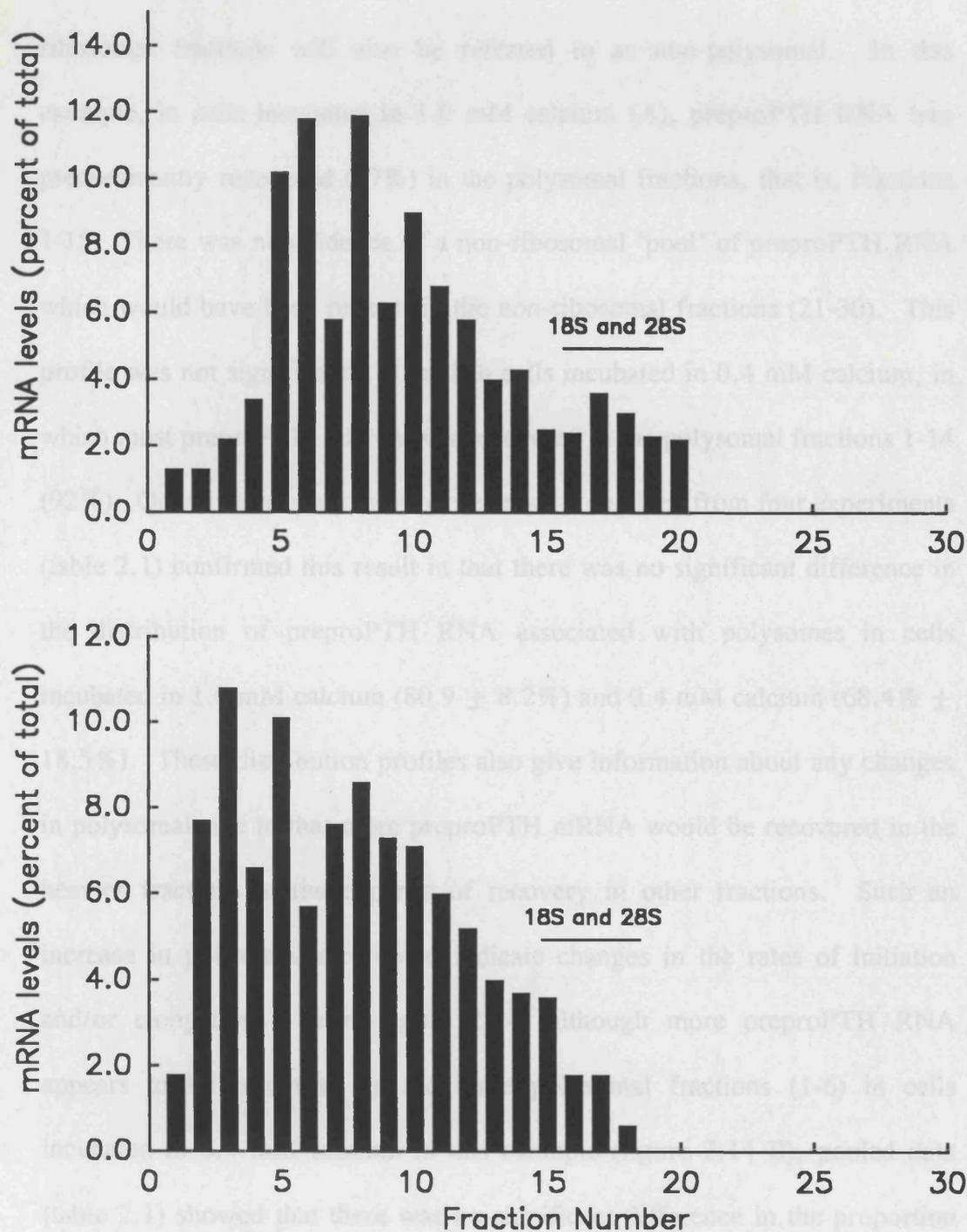


Figure 2.14. A typical example of a distribution profile after densitometric scanning of Northern blot of parathyroid cells incubated in 1.0 mM (upper panel) and 0.4 mM (lower panel) calcium for 48 hours expressed as a percentage of total following sucrose gradient ultracentrifugation. Fractions containing the 18S and 28S ribosomal RNA are indicated by the solid line.

the lightest fractions were designated non-ribosomal. The ribosomal and non-ribosomal fractions will also be referred to as non-polysomal. In this example, in cells incubated in 1.0 mM calcium (A), preproPTH RNA was predominantly recovered (87%) in the polysomal fractions, that is, fractions 1-15. There was no evidence of a non-ribosomal 'pool' of preproPTH RNA which would have been present in the non-ribosomal fractions (21-30). This profile was not significantly altered in cells incubated in 0.4 mM calcium, in which most preproPTH mRNA was recovered in the polysomal fractions 1-14 (92%). Quantitative analysis of pooled results obtained from four experiments (table 2.1) confirmed this result in that there was no significant difference in the distribution of preproPTH RNA associated with polysomes in cells incubated in 1.0 mM calcium ($80.9 \pm 8.2\%$) and 0.4 mM calcium ($68.4\% \pm 18.5\%$). These distribution profiles also give information about any changes in polysomal size in that more preproPTH mRNA would be recovered in the heavier fractions at the expense of recovery in other fractions. Such an increase in polysomal size could indicate changes in the rates of initiation and/or elongation. From figure 2.14, although more preproPTH RNA appears to be recovered in the large polysomal fractions (1-6) in cells incubated in 0.4 mM calcium in this example (figure 2.14 B), pooled data (table 2.1) showed that there was no significant difference in the proportion of preproPTH RNA recovered in fractions 1-6 in cells incubated in 1.0 and 0.4 mM calcium ($31.5\% \pm 9.9\%$ and $33.8\% \pm 4.1\%$, respectively). In addition, there was no significant difference whether fractions 1-5 or 1-7 were assessed. Thus, low calcium does not appear to increase polysomal size.

Calcium concentration (mM)	PreproPTH RNA (percentage of total)	
	<i>Polysomal</i>	<i>Post-Polysomal</i>
0.4	68.4 ± 18.5	31.6 ± 18.5
1.0	80.9 ± 8.2	19.1 ± 8.2
	<i>Fractions 1-6</i>	<i>Fractions 7+</i>
	0.4	31.5 ± 9.9
1.0	33.8 ± 4.1	66.2 ± 4.1

Table 2.1 illustrating the distribution of preproPTH mRNA between polysomal and non-polysomal fractions in parathyroid cells incubated in 0.4 or 1.0 mM calcium.

DISCUSSION

The data from this study have confirmed that low calcium did not change steady-state preproPTH mRNA levels in cells incubated in 0.4 or 1.0 mM calcium after 24 or 48 hours. Moreover, there was no difference in the 'apparent' half-life of preproPTH mRNA in cells incubated in 0.4 and 1.0 mM calcium in the presence of the transcriptional inhibitor, actinomycin D. However, low calcium increased membrane-bound preproPTH mRNA levels by two-fold in the absence of an overall change in steady-state preproPTH mRNA levels. This value is comparable with previous results which have shown that low extracellular calcium increased PTH secretion by 2-fold in bovine cells with no significant change in steady-state preproPTH mRNA levels (Brookman *et al.* 1986). Furthermore, this increase in polysomal association was enhanced in the presence of actinomycin D at a concentration which inhibited gene transcription, indicating a post-transcriptional site of regulation of PTH synthesis. The possibility that an 'untranslatable' pool of preproPTH mRNA exists in cells bound to RNPs or proteins inhibiting translation was investigated. There was no evidence for the existence of such a pool of significant proportions in the post-ribosomal fractions following fractionation of total RNA by sucrose gradient ultracentrifugation. This suggests that preproPTH mRNA detected in the polysomal supernatant exists predominantly bound to polysomes and ribosomes and not complexed as mRNP (Hawa *et al.* 1993).

As discussed earlier in chapter 1, there are a number of sites at which post-transcriptional regulation can occur including prolonging or reducing the

half-life of mRNA, increasing the level of mRNA available for translation by recruitment of existing mRNA from an 'untranslatable' pool, and altering the rates of initiation and/or reinitiation of mRNA onto polysomes and the rate of elongation of polypeptide chains.

Regulation by each of these mechanisms has been reported for a range of hormones and proteins, for example, Paek & Axel (1987) have reported that glucocorticoids enhance the stability of human growth hormone (hGH) mRNA by increasing the length of poly(A) tract. At maximal induction, hGH mRNA was approximately 100 nucleotides longer than in control cells. The mechanism is currently unknown, however, the authors have suggested that the increase in length may be due to a decrease in the rate of degradation of the poly(A) tail or a time-dependent addition of adenylate residues to the 3' end of the cytoplasmic mRNA. More recently, Murphy *et al.* (1992) have shown that GH mRNA transcripts in the anterior pituitary of thyroid-depleted rats have poly(A) tails which are 100-150 bases longer than in euthyroid rats. This yields GH mRNA with a half-life of approximately 10-12 hours from thyroid hormone-depleted rats compared to 5 hours in euthyroid rats. The change in size may be due to differential polyadenylation of the GH transcript in the nucleus of euthyroid or hypothyroid rats or due to a difference in the rate of degradation of the poly(A) tract. Another example of increased stability of mRNA has been reported by Gellersen *et al.* (1992) who demonstrated that retinoic acid increased prolactin secretion with a concomitant increase in mRNA levels in B-lymphoblastoid IM-9 cells. The authors suggested that in the presence of retinoic acid, transcripts were

preferentially associated with membrane-bound polysomes and thus protected from degradation.

The existence of an 'untranslatable' pool of mRNA has been well documented with respect to ferritin synthesis. Iron administration to rats increases hepatic ferritin synthesis but has little effect on ferritin gene transcription (Aziz & Munro, 1986) and further investigation revealed the presence of an 'untranslatable' pool of ferritin mRNA in the cytoplasm, which exists bound to RNPs or other proteins in iron-depleted cells. Iron administration induces recruitment of mRNA from this compartment onto actively translating polysomes thus increasing mRNA available for translation without altering gene transcription or steady-state mRNA levels. Another example of this type of regulation has been described in isolated rat pancreatic cells, in which glucose stimulated proinsulin synthesis in the absence of a change in proinsulin gene transcription (Itoh & Okamoto, 1980). A significant amount of proinsulin mRNA was detected in the post-polysomal fraction, that is, associated with RNPs and free mRNA and this fraction was decreased by glucose stimulation. The authors have suggested that regulation of proinsulin synthesis in pancreatic islets may be due to transfer of proinsulin mRNA from cytoplasmic mRNP to membrane-bound polysomes as well as greater translational efficiency. Subsequently, Welsh *et al.* (1986) have suggested that high glucose concentrations may also stimulate elongation rates of nascent preproinsulin and increase the transfer of initiated insulin mRNA molecules from the cytoplasm to microsomal membranes by a signal recognition particle-mediated (SRP) mechanism that involves the modification

of interactions between SRP and its receptor.

Whichever mechanism mediates translational control of PTH synthesis, a protein (or proteins) which interacts with preproPTH mRNA may be involved. This possibility is supported by the results from this study where actinomycin D enhanced the polysomal association of preproPTH mRNA. Such protein(s) may interact with the general translational machinery, however, since the effects of low calcium on PTH synthesis have been shown to be specific, it is more likely that the protein may interact directly with the 5'-UTR of preproPTH mRNA and alters initiation. For example, low calcium may dissociate the RNA-protein complex thus permitting initiation of translation and increasing polysomal content of preproPTH mRNA and PTH synthesis whilst in the presence of high calcium, the protein may remain bound and thus reduce translation. Conversely, these proteins may bind to the 3'-UTR to alter the half-life of mRNA on the polysome.

The existence of a 'calcium-sensitive' mRNA-binding, repressor protein(s) in parathyroid glands could explain observations in cultured parathyroid adenomas, in which there is a dissociation between the preproPTH mRNA levels and PTH synthesis (Farrow *et al.* 1988). For example, this protein may be less abundant or have a shorter half-life in adenomas when compared with normal cells, with a consequently higher basal rate of PTH synthesis. In addition, the protein may be less sensitive to changes in calcium concentration, such that higher concentrations are required to induce association between repressor protein and preproPTH mRNA and reduce translation. This may explain the higher 'set-point' (the concentration of

calcium causing half of the maximal inhibition of PTH release) reported in adenomatous tissue (Brown et al. 1979) compared to normal human and bovine glands.

Such proteins, which interact with ferritin mRNA to suppress translation have been partially characterised (Leibold & Munro, 1988; Walden et al. 1988), and the cDNA of a ferritin regulatory protein has been cloned (Rouault et al. 1990). These 'iron-sensitive' proteins bind to a 28 nucleotide (nt) conserved sequence, iron responsive elements (IRE) in the 5'-UTR of ferritin mRNA (Leibold & Munro, 1988) in response to low intracellular iron levels, with resultant inhibition of ferritin translation. Predicted RNA secondary structures show a potential stem-loop structure formed by the 28 nt sequence. Evidence suggests that the IRE-binding protein inhibits cap recognition or initial 40S ribosomal subunit binding at the 5'-end. Similar IRE have been described in the 3'-UTR of transferrin receptor mRNA (Casey et al. 1988), although in that case the interaction with protein increased the stability of the mRNA, possibly protecting the transcript from degradation. These transferrin receptor 3'-UTR sequences are also capable of folding into stem-loop structures that resemble the ferritin IRE. Proteins similar to the iron-sensitive proteins, which may interact with proinsulin mRNA to form mRNP, have been reported by Knight & Docherty (1992). They have demonstrated a series of proteins, using gel mobility shift assays and UV cross-linking, which bind to the 5'-UTR of proinsulin mRNA and identified potential stem-loop structures within this region.

In summary, the data in this study have demonstrated that in cultured

bovine cells, low calcium stimulates PTH synthesis via a post-transcriptional site. The increase in polysomal content of preproPTH mRNA induced by low calcium was not due to recruitment of mRNA from an inactive cytoplasmic pool. In addition, this increase does not yield larger polysomes and therefore is unlikely to be due to an increase in the rate of initiation/reinitiation relative to the rate of elongation, although changes in these cannot be entirely excluded since initiation and elongation rates were not directly determined in this study and would require the measurement of, for example, ribosome transit times. However, these results do indicate that low calcium probably increases the number of times a RNA molecule is translated thus increasing the apparent half-life of preproPTH mRNA associated with polysomes.

In conclusion, it is likely that regulation of PTH synthesis is due to a combination of changes in gene transcription and translation, with the latter providing a more rapid mechanism of control than the former, which provides a response to long-term changes.

CHAPTER 3

INTERACTION OF THE RECEPTOR FOR 1,25(OH)₂D₃ ON THE 5'-FLANKING REGION OF THE BOVINE PTH GENE

INTRODUCTION

SYNTHESIS AND ACTIONS OF VITAMIN D

Vitamin D was discovered as a consequence of rickets, a disease of the bone, which was common in central Europe after the First World War. At this time in Vienna, Dame Harriet Chick showed that rickets in children could be cured with cod liver oil or exposure to sunlight. Further studies led to the realisation that irradiation could yield vitamin D₃ and that it was important for the calcification of bone.

Vitamin D₃ can be synthesised in the skin from 7-dehydrocholesterol to cholecalciferol (vitamin D₃) as well as being obtained through the diet. This is the natural form of vitamin D₃, which is further metabolised in the liver microsomes, where it is hydroxylated at position 25 by 25-hydroxylase to give 25-hydroxycholecalciferol. This is the major form of vitamin D in the circulation which is then metabolised and activated in the kidney by hydroxylation in the 1-position by 1- α -hydroxylase, resulting in the biologically active form, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). The kidney is the major site of regulation of vitamin D activity and the 1- α -hydroxylase system is present in the mitochondria of the proximal convoluted tubules and proximal straight tubules. The activity of the 1- α -hydroxylase system is dependent on serum calcium and phosphate concentrations, with low calcium and PTH stimulating activity. Another circulating metabolite of vitamin D₃, formed from 25-hydroxycholecalciferol is 24,25-dihydroxycholecalciferol, synthesised by 24-hydroxylation in the kidney.

1,25(OH)₂D₃ has diverse biological effects in the body, both genomic actions as seen in the intestine, bone and kidney, in cell growth and differentiation as well as non-genomic actions in the intestine. The genomic actions of 1,25(OH)₂D₃ are mediated through intracellular protein receptors, VDR, which bind the hormone and interact with specific sites in target genes (HREs) resulting in changes in gene transcription of specific proteins such as osteocalcin (McDonnell et al. 1989), calbindin-D_{28K} (Christakos et al. 1989), c-myc (Simpson et al. 1989) and PTH (Cantley et al. 1985). These receptors are intracellular polypeptides of approximately 50 kDa which bind 1,25(OH)₂D₃ with high affinity (Hughes & Haussler, 1978). VDR have been identified and cloned from avian, human and rat sources (McDonnell et al. 1987; Baker et al. 1988; Burmester et al. 1988). The main action of 1,25(OH)₂D₃ is to stimulate calcium absorption in the intestinal mucosa by increasing the synthesis of a calcium binding protein (calbindin-D_{28K}) involved in transporting calcium across intestinal cells. Calbindin-D_{28K} binds calcium with high affinity and belongs to a gene superfamily which includes calmodulin and troponin C (Minghetti et al. 1988). 1,25(OH)₂D₃ has also been shown to regulate parathyroid gland activity suppressing preproPTH mRNA concentrations and PTH secretion in normal bovine glands in vitro (Cantley et al. 1985; Karmali et al. 1989) and in rat glands in vivo (Silver et al. 1986). In addition, using nuclear run-off assays, Sherwood et al. (1987) have demonstrated that this is a direct effect on the rate of PTH gene transcription. In contrast to the response of normal parathyroid tissue to 1,25(OH)₂D₃, PTH secretion from human parathyroid adenomata in vitro has

been found not to be suppressed by $1,25(\text{OH})_2\text{D}_3$ after 16 hours (Rudberg *et al.* 1984). Furthermore, this lack of response to $1,25(\text{OH})_2\text{D}_3$ was demonstrated even after 48 hours with both PTH secretion and mRNA levels remaining unchanged. This suggests insensitivity of tumours to regulation by the metabolite, however, this does not seem to be due to a lack of receptors but may be due to a defect in the interaction between the receptor and the PTH gene (Karmali *et al.* 1989).

$1,25(\text{OH})_2\text{D}_3$ also stimulates nongenomic biological responses, utilising a signal transduction pathway which is independent of the regulation of gene transcription. Under these circumstances the hormone is envisaged to bind a putative membrane recognition element which is then coupled to the opening of Ca^{2+} channels resulting in rapid biological responses. Nemere *et al.* (1984) have shown that $1,25(\text{OH})_2\text{D}_3$ can stimulate the rapid transport of $^{45}\text{Ca}^{2+}$ in chick intestine (within 2-4 minutes) and in addition, $1,25(\text{OH})_2\text{D}_3$ has been shown to open voltage-gated Ca^{2+} channels in rat osteosarcoma cells (ROS 17/2.8) (Caffrey & Farach-Carson, 1989). In both systems, the biological response appeared within seconds to minutes of administration of $1,25(\text{OH})_2\text{D}_3$. Protein kinase C and cAMP-dependent protein kinase may play a potential role in mediating $1,25(\text{OH})_2\text{D}_3$ activation of Ca^{2+} channels (deBoland & Norman, 1990).

In the study described here, the effects of $1,25(\text{OH})_2\text{D}_3$ on PTH gene transcription were investigated. $1,25(\text{OH})_2\text{D}_3$, as mentioned earlier, acts via its receptors, VDR, which are a member of a superfamily of ligand-inducible transcription factors including the receptors for steroid hormones, such as

glucocorticoids (GR), progesterone (PR), oestrogen (ER), and androgens (AR), as well as hormonal forms of vitamin A and D (Kumar & Chambon, 1988). Much of our knowledge of the function of VDR stems from the studies of steroid hormone receptors and hence, it is appropriate at this point to describe the structural organisation of steroid hormone receptors and the functions of the various domains.

STEROID HORMONE RECEPTORS

Like many transcriptional regulatory proteins, the steroid hormone receptors are single polypeptides that are organized into relatively discrete functional domains. Upon association with a ligand, the DNA binding region of nuclear receptors bind to specific DNA elements, HREs, located upstream of responsive genes, and up- or down-regulate transcription, by functionally interacting with other components of the transcriptional apparatus. HREs consist of two hexameric half-sites of DNA bases orientated as inverted repeats of AGGTCA seen in the oestrogen (ERE), glucocorticoid (GRE) and progesterone response elements (PRE), or as direct repeats of half-site sequences seen in VDRE as described in chapter 1. The half-site sequence of various nuclear receptors is frequently the same, however, the spacing between the sites differs.

The various functions of receptors, ligand binding, nuclear translocation, dimerisation, DNA binding, and transactivation have been assigned to particular amino acid sequences within the protein. The general organisation of these domains is shown in figure 3.1 and are described in more detail

below.

Ligand binding domain

This domain of steroid hormone receptors is approximately 25 kDa in size, moderately conserved and contains the domains for steroid binding and ligand-dependent transcriptional activation, which results in an increased affinity for the nucleus and DNA. The conserved amino acids of this region are important for the formation of a hydrophobic pocket and some of the non-conserved amino acids are essential for specific ligand binding. Nakajima *et al.* (1993) have demonstrated that cysteine residues within the hormone binding domain are important for high affinity binding of the ligand and may be critical to the transcriptionally active conformation of the receptor. The precise role of these cysteine residues is unclear, although their role in the formation of zinc fingers in the DNA binding domain is well known.

Steroid hormone receptor-mediated activation of target gene expression *in vivo* is known to be dependent upon ligand binding (O'Malley *et al.* 1979; Yamamoto, 1985; Beato, 1989). DNA binding activities of ER (Kumar & Chambon, 1988) and PR (Bagchi *et al.* 1990) have been shown to be influenced by their respective ligands. Steroid hormone receptors such as GR and ER are complexed to hsp90 (Denis *et al.* 1987) and ligand is believed to induce a transformation of the ER from the 8-10 S to a smaller active 4S form which can bind to its cognate response element. Green & Chambon (1988) have developed a model in which hsp90 binds to the ligand binding domain of unoccupied receptor and maintains it in a transcriptionally inactive state.

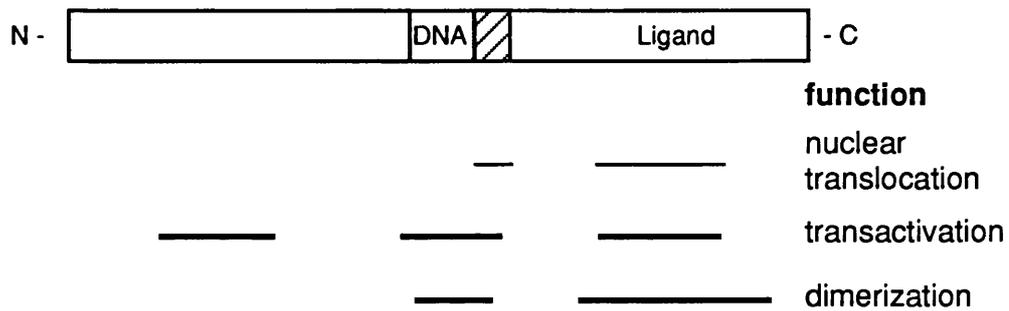


Figure 3.1. General structural and functional organisation of nuclear hormone receptors. The N-terminal region contains sequences that are involved in transactivation; the DNA binding domain contains transactivation and dimerisation functions, and the ligand binding domain is important for specific ligand binding as well as dimerisation. This figure has been adapted from Beato (1989).

Subsequent binding of ligand triggers the release of hsp90 from the receptor complex and reverses the repression. It is possible that in the ligand-free receptor, the DNA binding domain is not able to interact with DNA because it is masked by other domains of the proteins or by other components of the oligomeric protein complex. The region of the receptor involved in interactions with hsp90 has been mapped to the carboxy-terminal half (Howard et al. 1990). This may explain why the ligand binding domain is important for maintaining the inactive state of receptors in the absence of ligand, as truncated receptors lacking the ligand binding domain are constitutively active (Godowski et al. 1987). Whether receptors for $1,25(\text{OH})_2\text{D}_3$ are associated with heat shock proteins remains controversial, however, in this study it is possible that dissociation of the complex occurs during isolation of receptors and consequently, $1,25(\text{OH})_2\text{D}_3$ was not necessary for interaction with the PTH gene.

There have been several reports of ligand-independent transactivation but it is not clear whether in the absence of hormone the ligand-free receptor exerts any physiological role. For example, Matkovits & Christakos (1993) have demonstrated that ligand is not required for VDR-mediated activation of transcription and that treatment of transfected cells with $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M) or okadaic acid activated transcription. The presence of okadaic acid, a phosphatase inhibitor, permits phosphorylation of VDR and hence transactivation. In addition, Ross et al. (1993) have reported that VDR extracted from rats maintained on a vitamin D-deficient diet are capable of binding VDRE with initial VDR-DNA interaction occurring in a ligand-

independent manner and the addition of $1,25(\text{OH})_2\text{D}_3$ resulting in increased binding with greater affinity. Similarly, ligand has not been demonstrated to have an effect on the DNA binding capacity of thyroid (Damm *et al.* 1989) and retinoic acid (Hoffman *et al.* 1990), although this area remains controversial. In contrast, Liao *et al.* (1990) have suggested that the interaction of VDR with specific DNA is modulated in a concentration-dependent manner by $1,25(\text{OH})_2\text{D}_3$ although the unoccupied receptor is also capable of binding VDRE but with lower affinity. In addition, several studies have shown that binding of steroid hormone receptors to DNA requires prior activation of the receptor by, for example, salts (Buller *et al.* 1975) or binding of the hormone itself (Schauer *et al.* 1989).

The interaction of hormone receptors with each other, with DNA, and with other transcription factors is modulated not only by binding of the hormone ligand but also by a number of other mechanisms, such as phosphorylation as indicated above. It seems that phosphorylation is partly induced by ligand binding, however, a certain amount is observed in the absence of hormone (Orti *et al.* 1989; Denner *et al.* 1990). Members of the steroid nuclear receptor superfamily including the mammalian glucocorticoid receptors (Singh & Mougdil, 1985) and progesterone receptors (Hurd *et al.* 1989) are phosphorylated by cAMP-dependent protein kinase A *in vitro*. VDR have been shown to be phosphorylated in a number of species, including mouse (Pike *et al.* 1985) and chicken (Brown & DeLuca, 1990) primarily on the serine residues by protein kinase C. In addition, results from Jones *et al.* (1990) have indicated that human VDR is also phosphorylated by caesin kinase

II at the N-terminal border of the hormone binding domain.

The function of receptor phosphorylation is still under investigation and many functional roles have been suggested, including the regulation of steroid binding, DNA binding, specificity of DNA binding, nuclear localisation, association with heat shock proteins and association with auxilliary transcription factors. It is possible that phosphorylation may play a role in receptor turnover rate and recycling (Auricchio, 1989).

Nuclear translocation

The intracellular localisation of hormone receptors and their nuclear translocation upon ligand binding is controversial. Based on cell fractionation studies, the ligand-free receptors were originally thought to be localised in the cytoplasm and to translocate to the cell nucleus upon binding of the ligand (Jensen et al. 1968). Picard & Yamamoto (1987) have shown that GR accumulate in the nuclei in a hormone-dependent fashion and the involvement of the ligand binding domain was demonstrated by deletion of amino acid residues from this domain resulting in loss of localisation activity. Their experiments also revealed that receptor localisation was affected by cell culture conditions, in particular, the inclusion of phenol red and foetal calf serum in culture media. In the absence of serum and phenol red, the ligand-free receptors were mainly cytoplasmic, whereas the addition of either component or the ligand (dexamethasone) increased nuclear accumulation of receptors. More recent data suggest that GR are nuclear even in the absence of ligand (Brink et al. 1992).

Although conventional immunocytochemical methods indicated that VDR are mainly nuclear (Clemens et al. 1988), improved techniques suggest that they may be cytoplasmic (Barsony et al. 1990). Using immunocytology (avidin-biotin and phycoerythrin fluorophore) with microwave fixation (9-15 seconds), Barsony et al. (1990) demonstrated that in the absence of $1,25(\text{OH})_2\text{D}_3$, the receptors were mainly cytoplasmic in normal human fibroblasts cultured without serum and phenol red, but treatment with $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M) resulted in a rapid reorganisation of VDR, with clumping within 15-45s and intranuclear accumulation of VDR clumps within 1-3 minutes. Wheat germ agglutinin (which prevents protein transport through nuclear pores) blocked the translocation of VDR in the presence of $1,25(\text{OH})_2\text{D}_3$.

In addition, studies in a human leukaemia cell line (HL60) have shown that VDR reside in the cytoplasm and are translocated to the nucleus after hormone binding (Jakob et al. 1992). Hormone-receptor complexes do not accumulate in the nucleus, but have a high turnover, possibly due to the presence of a limited number of specific VDR binding sites. The unoccupied receptor is envisaged as a molecule with the transactivation domain(s) silent and the DNA binding fingers repressed or contorted such that optimal binding to vitamin D_3 responsive enhancer elements is not achieved. Binding of the $1,25(\text{OH})_2\text{D}_3$ ligand renders the DNA binding fingers competent for tight and specific DNA binding and is presumed to alter the conformation of the putative acidic/hydrophobic motifs saturated with serines. Hormone-elicited phosphorylation could create highly acidic patches on the receptor that attract

or complex with positive domains in transcription factors.

Dimerisation

Upon ligand binding, some steroid receptors are thought to act as dimeric transcription factors to activate or repress expression of nuclear target genes by binding to HREs. In contrast to vitamin D₃, thyroid, and retinoic acid receptors, the steroid hormone receptors such as oestrogen, glucocorticoid and progesterone do not appear to form heterodimers with other members of the nuclear receptor family, although interactions with other nuclear proteins have been reported (Schule & Evans, 1991). Analysis of binding of the ER to its ERE (Kumar & Chambon, 1988) has indicated that two receptor monomers bind to single palindromic ERE and that dimerisation results from interaction between regions located in both the DNA and ligand binding domains. Two dimerisation domains in receptors have been identified, a "strong" hormone-inducible dimerisation function in the ligand binding domain and a "weak" constitutive function associated with the DNA binding domain (Kumar & Chambon, 1988). This model is generally applicable, except that several receptors bind preferentially to direct repeats in which protein monomers are proposed to lie in a head-to-tail orientation on DNA rather than inverted repeats of half-sites in a head-to-head orientation. An additional domain downstream of zinc finger module 2 in the DNA binding domain of an orphan receptor, retinoid X receptor β (RXR β), termed the T box (Wilson *et al.* 1992), has been identified which is thought to be involved in protein-protein interactions. This region has been implicated in interactions with residues at

the tip of module 1 which orientates and stabilises the T-box of one monomer to make intermolecular contacts with module 2 of an adjacent bound monomer.

A region within the ligand-binding domain of the mouse ER that is required for both receptor dimerisation and high affinity DNA binding has been identified by Fawell *et al.* (1990). This domain overlaps the region essential for oestradiol binding and lies more than 250 amino acids from the DNA binding region. The critical residues in the domain lie within a heptad repeat of hydrophobic residues that is conserved in a similar position in other members of the nuclear receptor family. Single amino acid substitutions of selected residues in the N-terminal half, but not the C-terminal half of the heptad repeat, that is from arginine at residue 507 to isoleucine at 518, prevented dimerisation. Steroid binding was abolished by point mutations in the centre of the conserved region, implying that the steroid binding and dimerisation domains overlap (Fawell *et al.* 1990).

The ligand binding region participates not only in homodimer formation as in the ER and GR but may also mediate formation of dimers comprised of heterologous receptors such as retinoic acid (RAR) and thyroid hormone (TR) receptor heterodimers (Glass *et al.* 1989) or heterodimers comprised of specific receptors and nuclear proteins, such as RAR and with RXR (Burnside *et al.* 1990) and TR and 3,5,3'-triiodothyronine receptor auxiliary protein (TRAP). TR deletion analysis suggests that dimer formation is mediated by a region with the carboxy-terminal half of the TR as well as through a region immediately downstream of the two zinc-binding modules (Zhang *et al.* 1992).

VDR have also been shown to form heterodimers with a nuclear accessory factor (NAF) (Sone *et al.* 1991) in addition to RXR β (Yu *et al.* 1991).

DNA binding domain

This domain which consists of 66 - 68 amino acids is most conserved within the receptor family and comprises two zinc finger-like modules, each of which has been proposed to coordinate a zinc ion with conserved cysteine residues (Hollenberg & Evans, 1988). This motif shows similarity to the zinc finger motif of 5S ribosomal RNA transcription factor TFIIA (Klug & Rhodes, 1987), characterised by pairs of cysteines and histidines which tetrahedrally coordinate a zinc ion. In contrast, the nuclear receptor DNA binding domain is proposed to involve four cysteines in each of the two fingers to coordinate zinc.

In the DNA binding domain of GRs shown in figure 3.2, the first four cysteines chelate to Zn²⁺ ion, forming module 1 with its loop of 13 amino acids between cysteine 2 and cysteine 3 (Evans, 1988). Module 2 is formed by four cysteines with a loop of 12 amino acids and is separated from module 1 by a linker of 15-17 amino acids. These two regions represent independent structural and functional subdomains within the DNA binding domain. Umesono & Evans (1989) have identified two boxes in the DNA binding domains of steroid hormone receptors, one proximal (P) and one distal (D) as shown in the DNA binding domain of the GR in figure 3.2 (see legend). The P box follows cysteine 3 and includes the three amino acid cluster glutamic acid-glycine-cysteine-lysine-glycine (EGCKG) in finger module 1 of VDR.

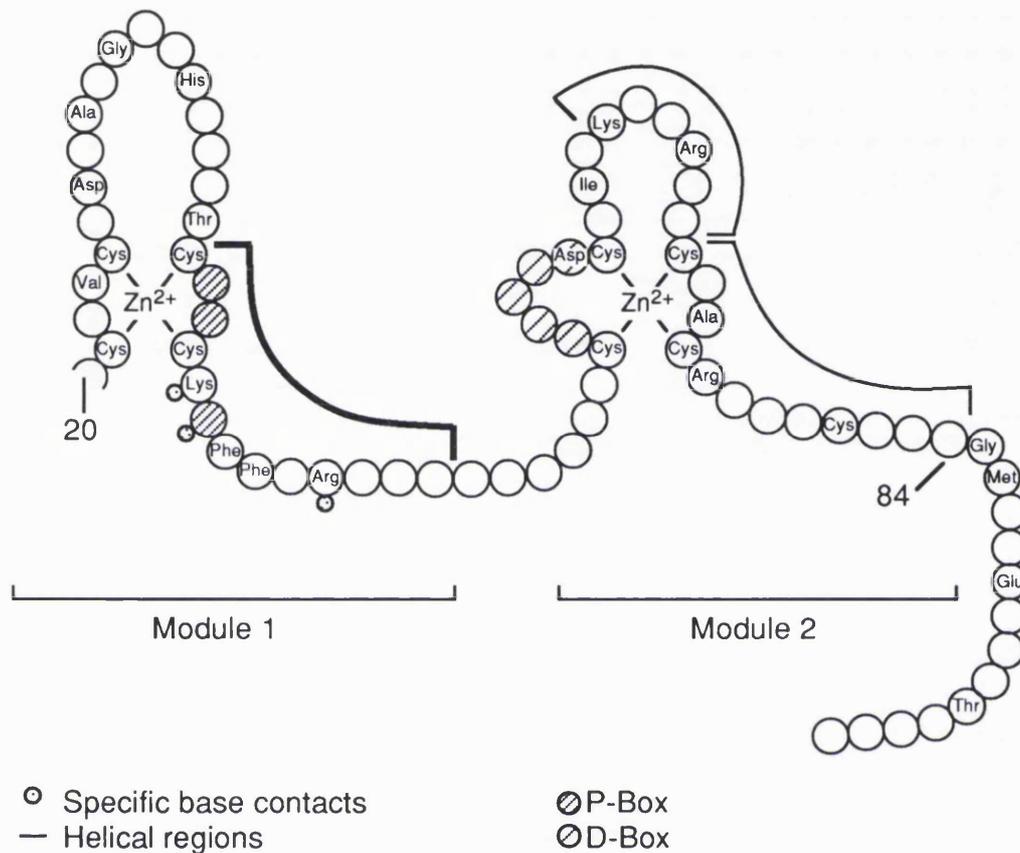


Figure 3.2. The glucocorticoid receptor DNA binding domain illustrating the two zinc finger-like modules. The α -helical region at the base of module 1 (indicated by the heavy line) is involved in making contacts with specific DNA bases in the major groove of each half-site of the HRE. The amino acid residues within the D and P boxes are highlighted. Homologous amino acids in the glucocorticoid and vitamin D₃ receptor are shown by the three letter abbreviation. This figure has been adapted from Umesono & Evans (1989).

This region contains a number of basic amino acids that are highly conserved and are predicted to be α -helical (figure 3.2, heavy line) and in direct contact with specific DNA bases in the major groove of each half-site of the HRE. The D box is located between cysteine 5 and cysteine 6 at the stem of module 2 and is important for protein - protein contacts in the receptor dimer that allow the correct spacing of monomers.

The DNA binding domain is solely responsible for specifying target gene activation. This was demonstrated by Green & Chambon (1987) using chimaeric receptors, in which the DNA binding domain of the ER was replaced with the corresponding region of the GR. This ER derivative activated a GRE-dependent, but ERE-independent reporter gene in response to oestradiol. In addition, by swapping individual ER fingers for GR, Green *et al.* (1988) demonstrated that the first finger module plays the major role in determining specificity of binding by making specific contact with some or all of the bases of the responsive element, although both fingers including a basic region immediately C-terminal to the second finger were required for DNA binding. In addition, mutagenesis experiments indicated that specific residues within the zinc finger region of the GR and ER were critical for DNA binding specificity (Mader *et al.* 1989; Danielsen *et al.* 1989; Umesono & Evans, 1989) and these results have been confirmed by three-dimensional structural analysis (Hard *et al.* 1990; Luisi *et al.* 1991). In particular, the crystal structure of GR DNA binding domain complexed to a GRE shows that residues making specific base contacts are localised to an α -helix at the carboxyl terminal end of the first module, and the amino acids essential for

DNA-induced dimerisation of two monomers lie in the D box (Luisi et al. 1991).

Members of the nuclear receptor superfamily fall into subgroups which recognize the same response element core, and the amino acid sequence of the P box can be used to predict core preference (Umesono & Evans, 1989). Receptors carrying the glycine-serine-cysteine-lysine-valine (GSCKV) motif at the amino terminus of the recognition α -helix such as GR, MR, PR and AR all recognize a GRE with high affinity. Receptors carrying the glutamic acid-glycine-cysteine-lysine-alanine (EGCKA) or glutamic acid-glycine-cysteine-lysine-glycine (EGCKG) motif at the corresponding position as in TR, ER, RAR and VDR all appear to bind to the ERE core (5'-AGGTCA-3') with high affinity. Although GREs can function virtually as efficiently as response elements for progesterone, androgen, and mineralocorticoid hormones, receptor specificity is conferred by the hormones eliciting distinct physiological effects in cells. This arises from tissue-specific expression of the corresponding receptors or the role of transcription/auxillary factors in directing specific gene activation, for example, GR has been shown to act synergistically with a variety of transcription factors, including SP1 and CTF/NFI (Strahle et al. 1988).

Regions required for hormone-dependent transcriptional activation

In the oestrogen receptor, two transcriptional activation functions (TAFs) have been identified using transient transfection experiments. TAF-1 in the N-terminal domain is constitutively active while TAF-2 requires a region in

the C-terminus of the hormone binding domain (Kumar et al. 1987; Lees et al. 1989). TAF-2 is conserved among many nuclear hormone receptors and is essential for hormone dependent stimulation of transcription. TAF-1 is conserved within the ER and GR, however, VDR lack a constitutive TAF-1 activity since they lack appreciable N-terminal sequences (Baker et al. 1988). As a consequence they may rely entirely on sequences in their hormone binding domains for transcriptional activation. Additional transactivation domains have been identified in the less well-conserved N-terminal region and also associated with the DNA binding domain.

AIMS OF THIS STUDY

The effects of $1,25(\text{OH})_2\text{D}_3$ on transcription of the PTH gene were investigated in this study since previous studies in this laboratory have shown that $1,25(\text{OH})_2\text{D}_3$ suppressed PTH gene transcription in bovine parathyroid glands in vitro via its receptors (Karmali et al. 1989). In addition, VDR had also been shown to interact with DNA sequences of target genes such as osteocalcin (McDonnell et al. 1989). Thus, binding sites for VDR on the 5'-flanking region of the bovine PTH gene were studied.

The interactions of receptors for $1,25(\text{OH})_2\text{D}_3$ on the 5'-flanking region of the bovine PTH gene were investigated using a combination of techniques including filter binding, Southwestern and gel mobility shift assays. Initial localisation of the binding site for VDR, VDRE, involved filter binding assays in which radiolabelled DNA fragments were incubated with proteins in solution, the resulting DNA-protein complexes were precipitated onto

nitrocellulose filters and the radioactivity retained on the filters was assessed. The amount of radioactivity is a reflection of proteins bound to radiolabelled DNA fragments. This assay is based on the selective retention of protein-DNA complexes but not free DNA by nitrocellulose filters. These assays have been used by Sastry & Kun (1988) to investigate the binding of poly (ADP-ribose) polymerase and histones to DNA.

Although the filter binding assay is a sensitive technique, it does not permit the exact determination i.e. molecular mass of the protein-DNA complexes. Thus, Southwestern assays (immobilised receptor assays) (Bowen *et al.* 1980) were used to define the binding site further. In these, DNA-binding proteins were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) on the basis of their molecular mass and transferred onto nitrocellulose filters. The filters were reacted with radiolabelled DNA fragments and visualised by autoradiography. Sastry & Kun (1988) have suggested that proteins in solution as in the filter binding assays, and those transferred to nitrocellulose filters, bind DNA in a similar manner. The advantage of this technique is that it permits the estimation of molecular size of proteins by gel electrophoresis and requires small amounts of radiolabelled probe. This technique has previously been used, for example, in determining the size of proteins interacting with the rat growth hormone gene promoter (Schaufele *et al.* 1990). To confirm the identity of the protein reacting with radiolabelled DNA fragments, receptor proteins were separated by SDS-PAGE, transferred to nitrocellulose filters and reacted with monoclonal antibodies to VDR. The identity of receptors for $1,25(\text{OH})_2\text{D}_3$ was confirmed by Western blotting

(immunoblotting) using monoclonal rat anti-VDR antibodies (9A7) to the receptor (Pike et al. 1987). The epitope of this antibody lies near the DNA binding domain of the receptor, just C-terminal to the zinc fingers (Allegretto et al. 1987) and thus could be used to demonstrate specificity of VDR.

To demonstrate protein-DNA interactions further, gel mobility shift assays which are based on electrophoretic separation of protein-DNA complexes from each other and from free DNA were used in this study. These assays were used to investigate the sequence specificity of binding by including a large excess of unlabelled DNA fragments in the binding reaction. In addition, the possible involvement of other proteins such as transcription factors AP1, AP2, AP3 and SP1 with VDR were also investigated using gel mobility shift assays. Several groups have shown the presence of binding sites for transcription factors within or adjacent to steroid response elements, for example, Schule et al. (1990) have shown the presence of an AP1 binding site within the VDRE in the human osteocalcin gene and suggested that the Fos-Jun protein complexes may be involved in VDR interaction with the gene. GR have been shown to be able to act synergistically with a variety of transcription factors, including CTF/NF1, SP1, and the CACC box binding factors (Schule et al. 1988). In addition, Diamond et al. (1990) have characterised a glucocorticoid response element of the mouse proliferin gene which serves as a binding site for the Fos-Jun heterodimer of the AP1 nuclear transcription factor complex and glucocorticoid receptor. In the presence of the c-fos, glucocorticoids exert a negative effect on proliferin gene transcription.

For clarity, the rest of this chapter will be divided into three parts and each

will contain a methods and a results section followed by a general discussion:

Part I) Localisation of VDRE within the 5'-flanking region

Part II) Further definition of VDRE

Part III) Characterisation of protein-DNA interactions

PART I

Localisation of VDRE within the 5'-flanking region

To localise the binding sites for the receptor for 1,25(OH)₂D₃, filter binding assays were used in which DNA fragments generated from the 5'-flanking region of the bovine PTH gene were incubated with VDR prepared from bovine parathyroid glands.

METHODS

All restriction enzyme buffers, DNA loading buffer, and general buffers are defined in Appendix II.

Generation of DNA fragments for filter binding assays

The bovine parathyroid hormone gene (PTH) consists of three exons (I, II and III) two intervening sequences (introns), the 5'-flanking and 3'-flanking regions (figure 3.3). The 5'-flanking region was digested with EcoRI and PvuII from a genomic clone and a 1750 bp region was subcloned into the EcoRI site of the plasmid vector, pGEM4. This region contains restriction sites for PstI and was used for subsequent digestions in binding studies.

To generate DNA fragments, the -1700 to +50 bp region in pGEM4 (80 µg) was digested with PstI (10 Units/µg) and PvuII (7.5 U/µg) in buffer H and spermidine (5 mM) at 37°C for 16 hours followed by termination of reaction with the addition of 0.1 volume DNA loading buffer. The DNA

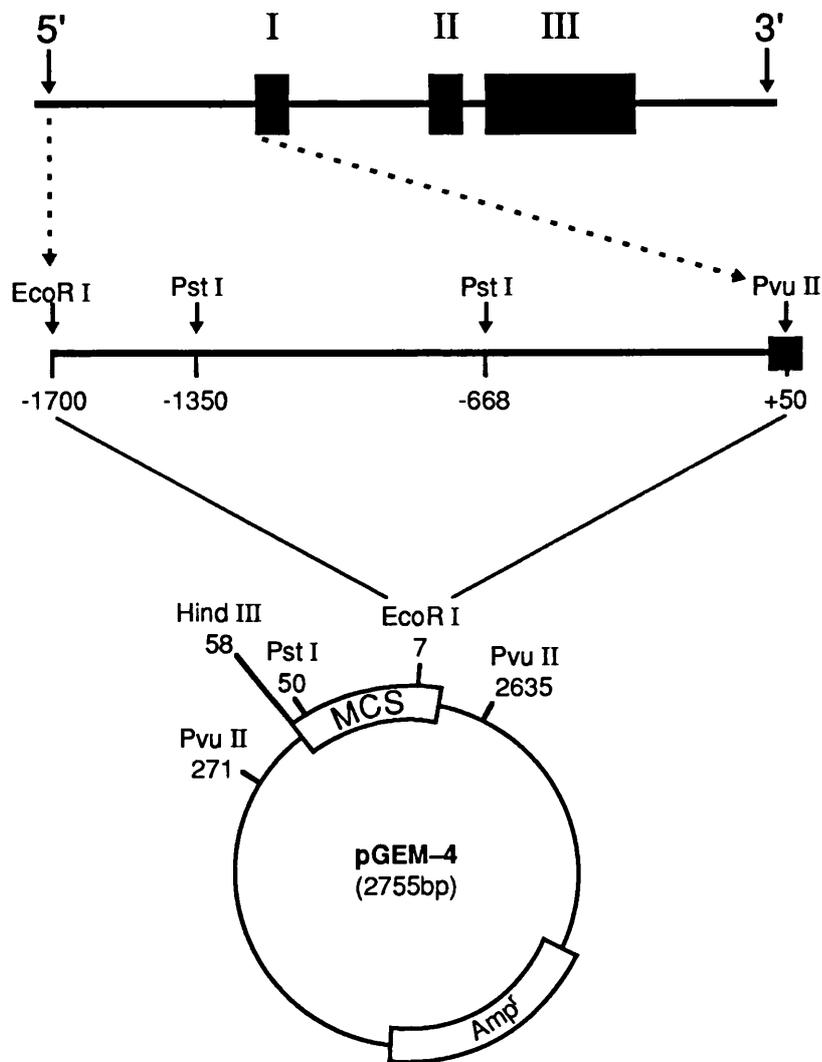


Figure 3.3. The -1700 to +50 bp fragment of the promoter region of the bovine PTH gene was digested with EcoRI and PvuII from a genomic clone and ligated into the EcoRI site of pGEM4 plasmid. The multicloning site contains a number of restriction sites including PstI and HindIII.

fragments were separated by electrophoresis in 1 X TAE buffer containing ethidium bromide (1.25 $\mu\text{g/ml}$) at a constant voltage of 60 V and purified by GeneClean or Mermaid kits (BIO 101 Inc. La Jolla, Ca, USA) as described in Appendix I.

Digestion of the -1700 to +50 bp region in pGEM4 yielded five fragments (figure 3.4) at approximately 2400, 720, 680, 470 and 220 bp (faint band on gel). The 720 and 680 bp fragments are quite similar in size spanning the regions -668 to +50 bp and -1350 to -669 bp indicated by the A and B respectively, in figure 3.4 lanes 1-4.

Since the region between -668 to -510 bp had not been sequenced previously and the enzyme restriction sites not known, to confirm the identity of these fragments, test digestion were performed using some enzymes which may digest the -668 to +50 bp region. Each DNA fragment (500 ng) was digested with either PstI + PvuII, HindIII, RsaI or Sau3A (0.02 Units/ng) in relevant buffers at 37°C for 16 hours. Lane 1 and 6 are undigested fragments used as controls (figure 3.5). Not unexpectedly, digestion of both bands with PstI + PvuII had no effect (lanes 2 and 7,) since there are no restriction sites within these regions. Digestion of band A with HindIII which is a diagnostic test, gave a fragment of approximately 600 bp (lane 3) and is consistent with the location of this site within the -668 to +50 bp region. Digestion with Sau3A (lane 5) gave two fragments of 700 and 560 bp, of which the 700 bp fragment is undigested DNA, whilst digestion with RsaI yielded a fragment of 540 bp. As regards band B, although digestion with RsaI and Sau3A yielded fragments of approximately 540 and 480 bp respectively, HindIII had

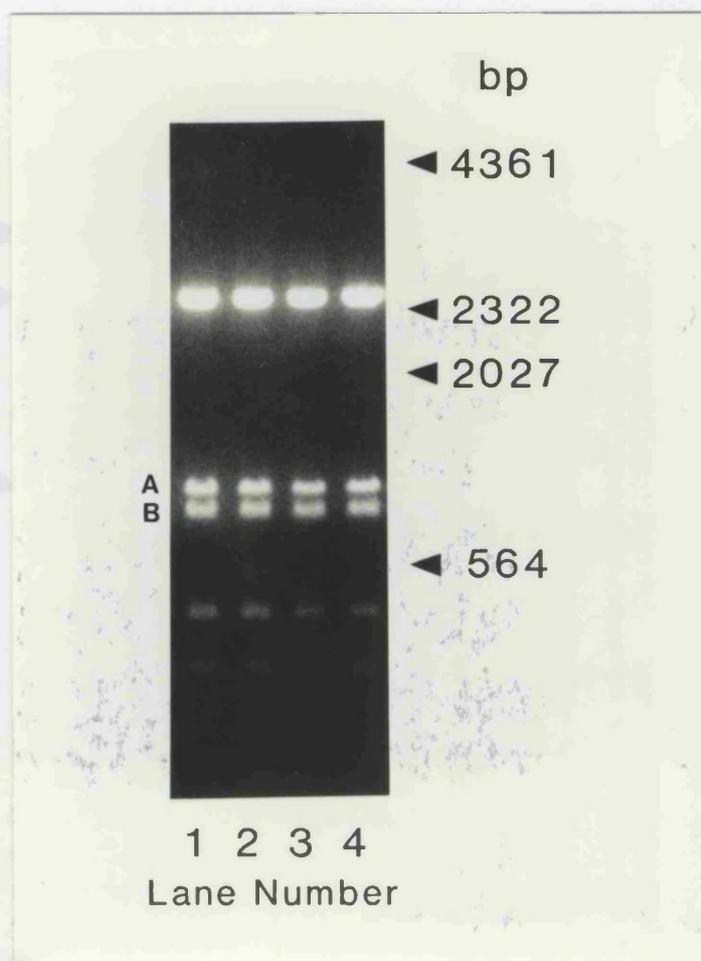
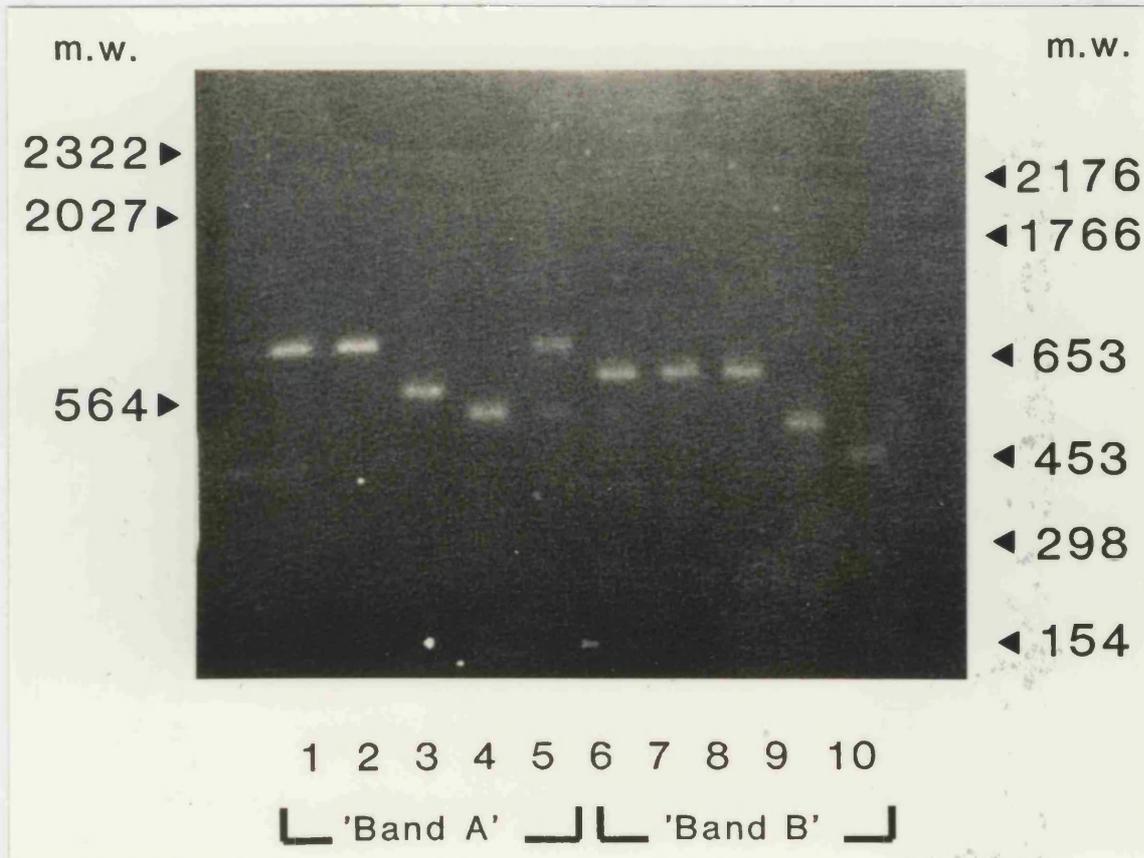


Figure 3.4. The -1700 to +50 bp region in pGEM4 was digested with PstI and PvuII to yield five fragments including the regions -668 to +50 bp and -1350 to -669 bp indicated by A and B, respectively. Molecular size markers are indicated on the right.

no effect indicating that this region was not the -658 to +50 bp fragment. Thus, these results confirmed that the -658 to +50 bp fragment was the upper of the two bands (A) obtained as a result of digestion of the -1700 to +50 bp region in pGEM4 with PstI and PvuII.

The -658 to +50 bp fragment was subsequently used for digestion with Sau3A (the restriction site is shown in figure 3.6 A) with 16 U Sau3A/ μ g



homogenised in 10mM Tris-HCl (pH 7.4), 10mM sodium molybdate, 20mM KCl, 1.5mM EDTA, 1mM DTT and 1mM phenylmethylsulphonyl fluoride (PMSF) (see 3.1). The homogenate was centrifuged at 1500 g for 15 minutes and then at 100 000 g for 1 hour (MSD superspeed 85

Figure 3.5. Test digestions of the two fragments generated by the digestion of -1700 to +50 bp fragment in pGEM4 with PstI and PvuII. Lanes 1 - 5 are digestions of 'band A' and lanes 6 - 10 are digestions of 'band' B. Lanes 1 and 6 represent undigested fragments, 2 and 7 with PstI and PvuII, lanes 3 and 8 with HindIII, lanes 4 and 9 with RsaI and lanes 5 and 10 with Sau3A. Molecular size markers are indicated.

no effect, indicating that this region was not the -668 to +50 bp fragment. Thus, these results confirmed that the -668 to +50 bp fragment was the upper of the two bands (A) obtained as a result of digestion of the -1700 to +50 bp region in pGEM4 with PstI and PvuII.

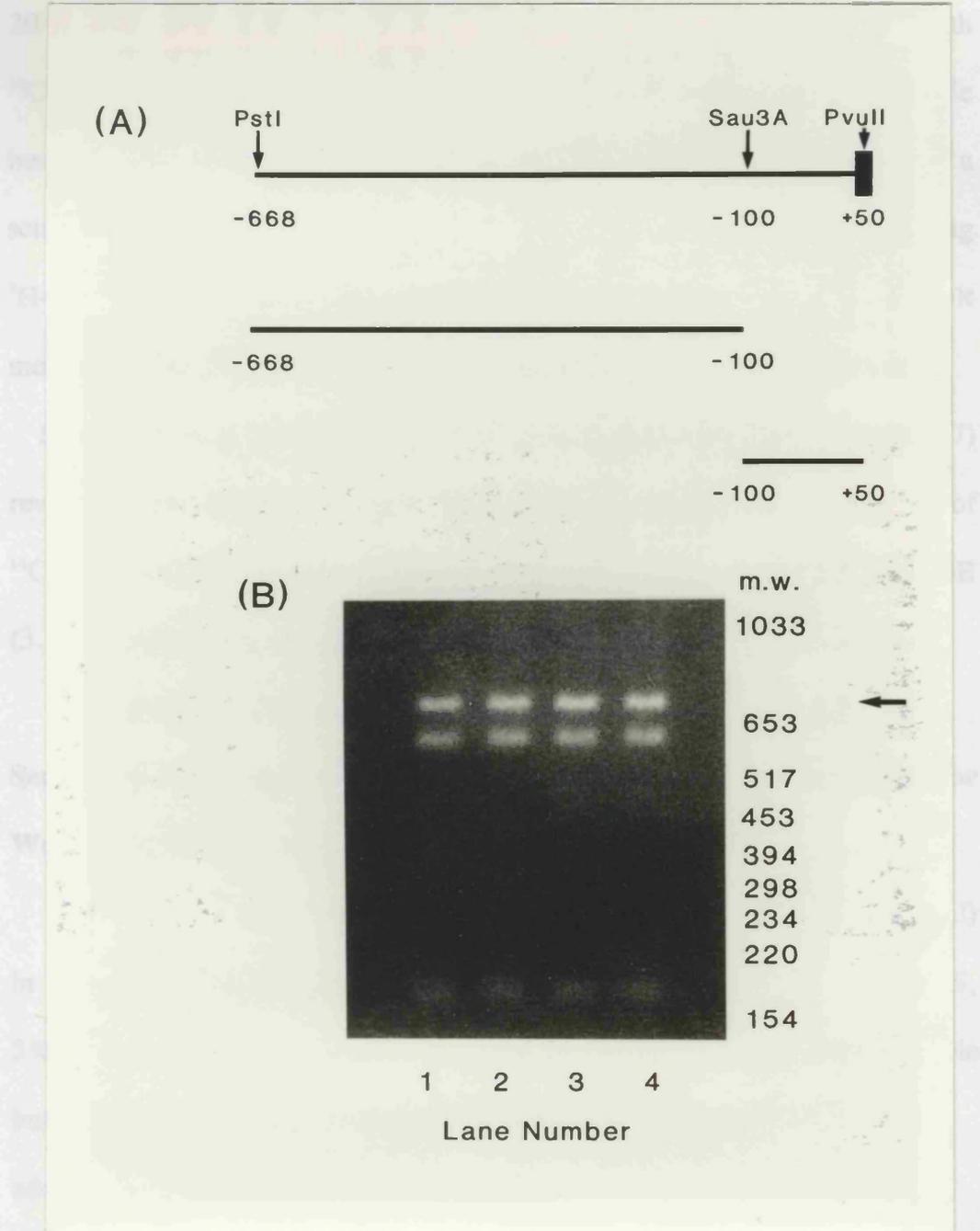
The -668 to +50 bp fragment was subsequently used for digestion with Sau3A (the restriction site is shown in figure 3.6 A) with 16 U Sau3A/ μ g DNA in buffer A at 37°C for 16 hours. This yielded three fragments of 700, 600 and 180 bp (faint band) representing undigested -668 to +50 (indicated by the arrow), -668 to -100 and -100 to +50 bp, respectively.

The bovine preproPTH cDNA was used as a control since VDR were not expected to bind to this region. The cDNA (10 μ g) which contains 345 residues of the coding region, 102 residues of 5' noncoding region and 23 residues of 3' noncoding region (470 bp) in pGEM4 was digested with PstI in buffer H at 37°C for 16 hours.

Preparation of receptors for 1,25(OH)₂D₃

Partially purified receptors were prepared from bovine parathyroid glands homogenised in 10mM Tris-HCl (pH 7.4), 10mM sodium molybdate, 300mM KCl, 1.5mM EDTA, 1mM DTT and 1mM phenylmethylsulphonyl fluoride (PMSF) (buffer A). The homogenate was centrifuged at 1500 g for 15 minutes and then at 100 000 g for 1 hour (MSE superspeed 65 ultracentrifuge). The resulting supernatant was incubated with ³H-1,25(OH)₂D₃ (4 nM) for 16 hours at 4° C followed by separation of free label with dextran-coated charcoal. Aliquots (0.5 ml) were layered onto 4 X 3.6ml

... gradient (5 - 20% w/v) in buffer A and centrifuged at 270000g for



... buffer (50% w/v) were loaded with the parathyroid cell extract only a 15%

Figure 3.6. Restriction sites within the -668 to +50 bp fragment for the enzyme Sau3A are shown in A and digestion of this region yielded two fragments spanning -668 to -100 and -100 to +50 bp. Undigested -668 to +50 bp fragment is indicated by ←. Molecular size markers are indicated on the right.

... min. Type-IVCL 2. 4. 10. 20. 30. 40. 50. 60. 70. 80. 90. 100. 110. 120. 130. 140. 150. 160. 170. 180. 190. 200. 210. 220. 230. 240. 250. 260. 270. 280. 290. 300. 310. 320. 330. 340. 350. 360. 370. 380. 390. 400. 410. 420. 430. 440. 450. 460. 470. 480. 490. 500. 510. 520. 530. 540. 550. 560. 570. 580. 590. 600. 610. 620. 630. 640. 650. 660. 670. 680. 690. 700. 710. 720. 730. 740. 750. 760. 770. 780. 790. 800. 810. 820. 830. 840. 850. 860. 870. 880. 890. 900. 910. 920. 930. 940. 950. 960. 970. 980. 990. 1000.

sucrose gradients (5 - 20% w/v) in buffer A and centrifuged at 270000g for 20 hours at 4°C in a swing-out rotor (MSE superspeed 65 ultracentrifuge) with ¹⁴C-ovalbumin as a marker. Fractions (0.5 ml) were collected using a needle inserted into the bottom of the tubes and ¹⁴C and ³H were counted on a scintillation counter. The presence of receptors in peak fractions containing ³H-1,25(OH)₂D₃ was confirmed by Western blotting using anti-VDR monoclonal antibodies (9A7). Fractions were pooled and frozen at -70°C.

Sucrose density gradient profile of partially purified VDR (figure 3.7) revealed a single peak corresponding to VDR (x---x) coincident with that of ¹⁴C-ovalbumin (o---o), which has a similar sedimentation coefficient as VDR (3.67 S) (Hughes & Haussler, 1978).

Separation of proteins by SDS-polyacrylamide gel electrophoresis for Western blotting

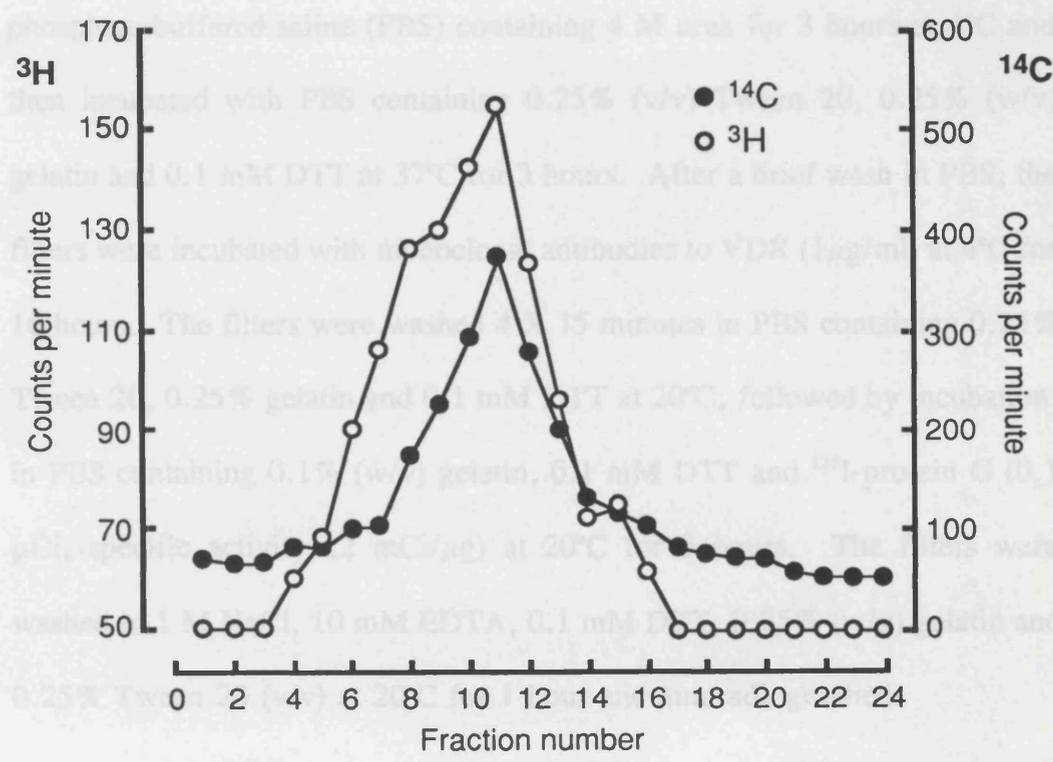
Partially purified receptors (50 μl) or bovine parathyroid cytosol (50 μl) in 15μl 0.5 M Tris-HCl (pH 6.0) containing 10% glycerol, 2% (w/v) SDS, 5% β-mercaptoethanol (v/v) and 0.05% (w/v) bromophenol blue (sample buffer X4) were boiled for 5 minutes, cooled on ice and 2.5 μl glycerol added. Molecular weight markers (14 300 to 200 000 Daltons) in sample buffer (X4) were loaded with the parathyroid cell extract onto a 15% polyacrylamide gel. Electrophoresis was performed in 50 mM Tris-HCl (pH 8.3), 0.38 M glycine and 0.1% SDS buffer at 100 V for 7 hours.

After electrophoresis, the gel and nitrocellulose filter were soaked in 25 mM Tris-HCl, 0.38 M glycine and 0.02% SDS buffer for 20 minutes at 20°C.

Transfer was performed in an LKB Multiphor ZI Novablot unit at 200 mAmps for 1.5 hours.

Western blotting (immunoblotting)

Following electroblotting, nitrocellulose filters were incubated in



Filter binding assays

DNA fragments -1350 to -669, -668 to +50, -668 to -100 bp fragments and 470 bp of the bovine FTH cDNA (20 µg of each) were dephosphorylated and radiolabelled using T₄ polynucleotide kinase as described in Appendix L and incubated with 20, 40, 100 and 200 µg pooled bovine parathyroid tissue

Figure 3.7. Purification of bovine receptors for 1,25(OH)₂D₃ by sucrose density gradient ultracentrifugation. VDR elutes as a single peak (○—○) coincident with that of ¹⁴C-ovalbumin (●—●).

Transfer was performed in an LKB Multiphor II Novablot unit at 200 mAmps for 1.5 hours.

Western blotting (Immunoblotting)

Following electroblotting, nitrocellulose filters were incubated in phosphate-buffered saline (PBS) containing 4 M urea for 3 hours at 4°C and then incubated with PBS containing 0.25% (v/v) Tween 20, 0.25% (w/v) gelatin and 0.1 mM DTT at 37°C for 3 hours. After a brief wash in PBS, the filters were incubated with monoclonal antibodies to VDR (1µg/ml) at 4°C for 16 hours. The filters were washed 4 X 15 minutes in PBS containing 0.25% Tween 20, 0.25% gelatin and 0.1 mM DTT at 20°C, followed by incubation in PBS containing 0.1% (w/v) gelatin, 0.1 mM DTT and ¹²⁵I-protein G (0.1 µCi, specific activity 12 mCi/µg) at 20°C for 3 hours. The filters were washed in 1 M NaCl, 10 mM EDTA, 0.1 mM DTT, 0.25% (w/v) gelatin and 0.25% Tween 20 (v/v) at 20°C for 1 hour and autoradiographed.

Filter binding assays

DNA fragments -1350 to -669, -668 to +50, -668 to -100 bp fragments and 470 bp of the bovine PTH cDNA (20 ng of each) were dephosphorylated and radiolabelled using T4 polynucleotide kinase as described in Appendix I, and incubated with 20, 40, 100 and 200 µg pooled bovine parathyroid receptor protein (prepared as described earlier) in 25 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.5 mM DTT, 150 mM KCl containing 1µg poly (dI-dC).poly (dI-dC) and 20 mg/ml bovine serum albumin (BSA) for 30 minutes at 20° C. Samples

were filtered through nitrocellulose filters (0.45 μm) pre-wetted with the above buffer and washed with 5 ml of the above buffer containing 5% dimethyl sulphoxide (DMSO). The radioactivity retained on the filters was measured on a scintillation counter and expressed as a percentage of control. Statistical significance was determined using a non-parametric test (Wilcoxon) and all results are expressed as mean \pm S.D.

RESULTS

Western blotting

This was used to verify the identity of the receptors for $1,25(\text{OH})_2\text{D}_3$ in peak fractions pooled after sucrose density gradient ultracentrifugation. Figure 3.8 shows autoradiographs of Western blots of filters containing partially purified receptors for $1,25(\text{OH})_2\text{D}_3$ (lane 1) and parathyroid cytosol (lane 2). A single band at a molecular mass of approximately 50 000 Da indicating the presence of VDR was detected in both preparations. Standard protein molecular size markers are indicated on the right.

Filter binding assays

Incubation of radiolabelled DNA fragment -668 to +50 bp with 20, 40, 100 and 200 μg of protein in the absence of poly(dI-dC).poly(dI-dC) resulted in significant retention of radioactivity on the filter (figure 3.9 A). Retained radioactivity increased from $180 \pm 47\%$ to $424 \pm 19\%$ (mean \pm SD) of control with 20 μg and 200 μg protein respectively. The addition of poly(dI-dC).poly(dI-dC) in the reaction mixture which reduces non-specific binding, abolished binding at 20 μg and 40 μg protein. However, significant and specific binding remained on the filters with 100 μg and 200 μg at $188 \pm 22\%$ and $288 \pm 63\%$ of control, respectively.

In contrast, incubation of the -1350 to -669 bp fragment with protein in the absence of poly(dI-dC).poly(dI-dC) increased radioactivity retained on the filter from $148 \pm 21\%$ with 20 μg protein rising to $473 \pm 3\%$ of control with

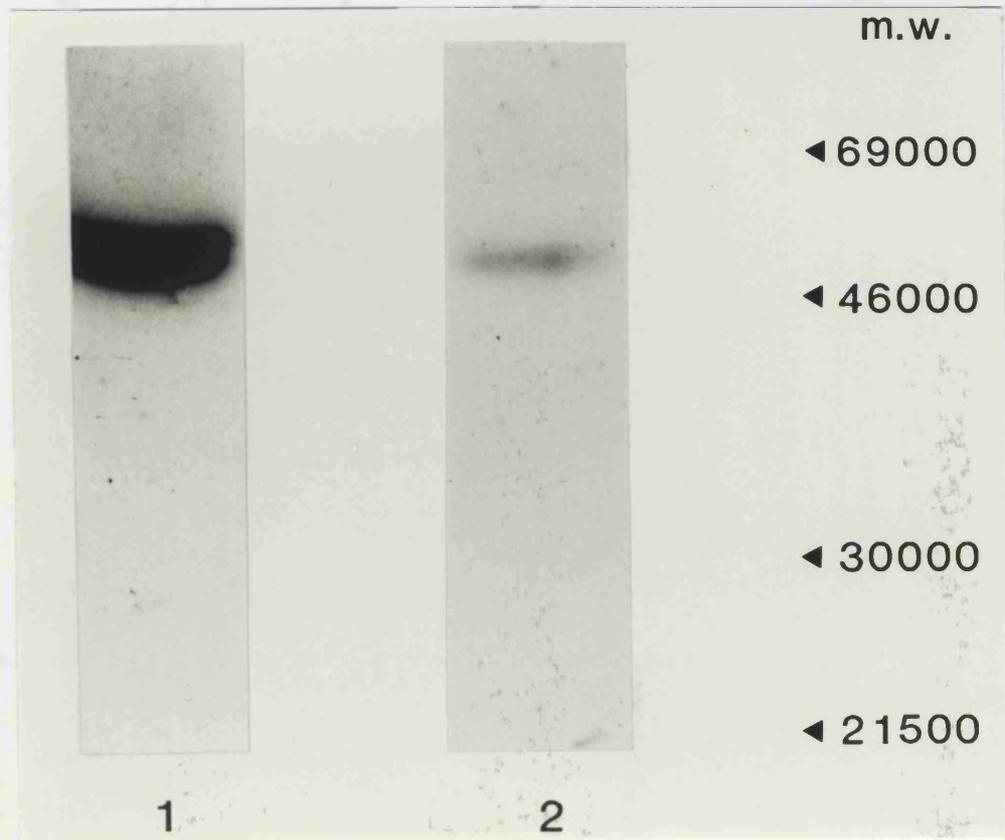


Figure 3.8. Autoradiographs following SDS-PAGE and reaction with monoclonal antibodies in Western blots of partially purified receptors for $1,25(\text{OH})_2\text{D}_3$ (lane 1) and parathyroid cytosol (lane 2). Standard protein molecular size markers are indicated on the right.

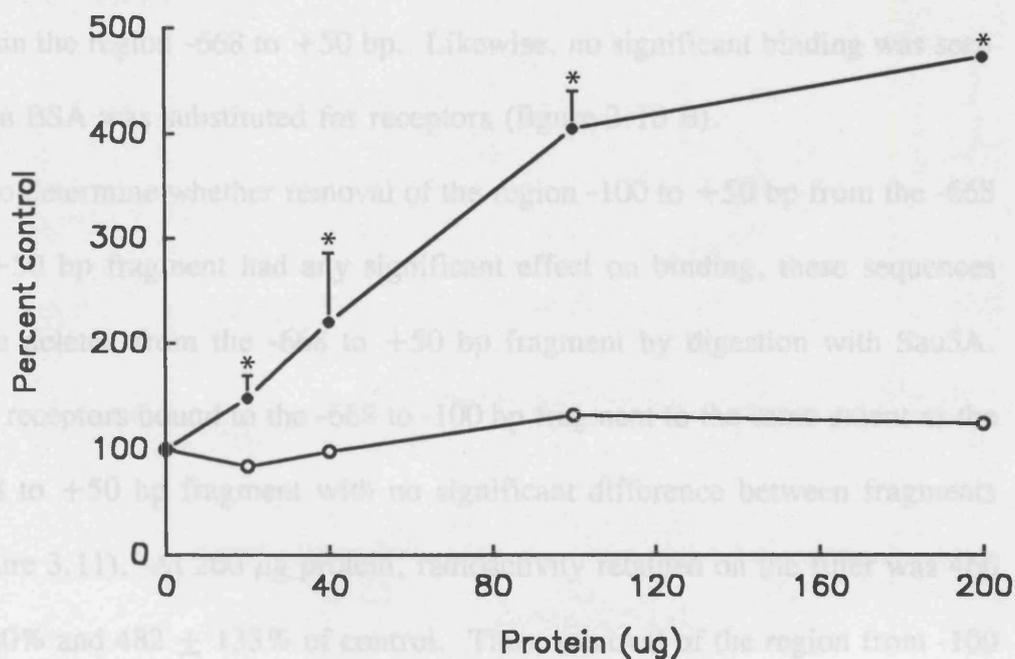
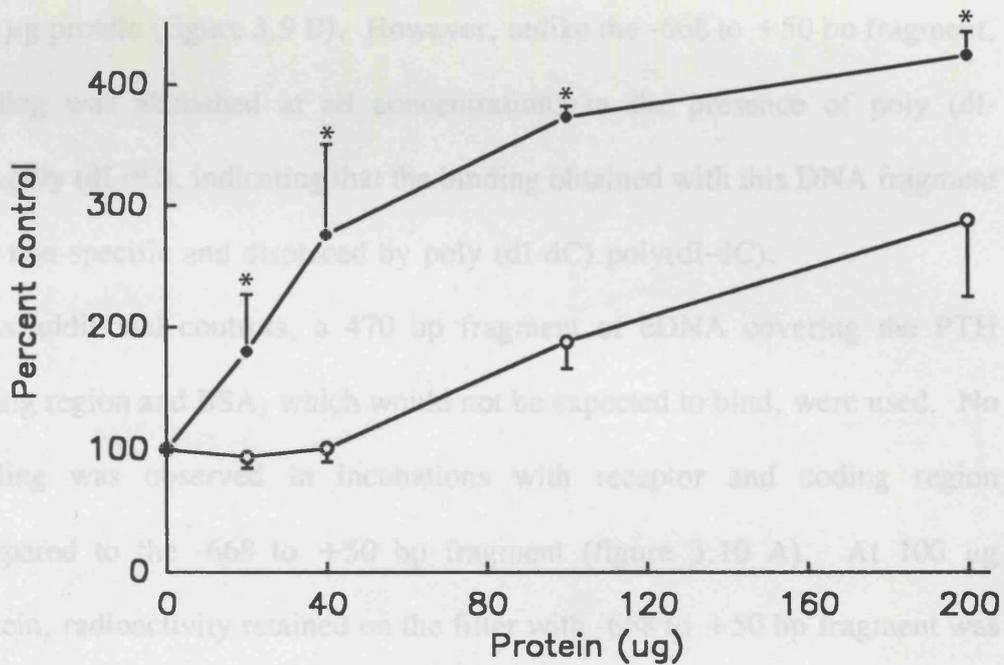


Figure 3.9 shows the effect of poly (dI.dC).poly (dI-dC) on binding of the -668 to +50 bp fragment (A) and the -1350 to -669 bp fragment (B) to VDR. Radiolabelled DNA fragments were incubated with receptor protein (0 - 200 μ g) in binding buffer in the presence (○---○) and absence (●---●) of poly(dI.dC).poly(dI-dC). Results are represented as mean \pm SD with * $p < 0.01$. SD obtained in the absence of poly(dI.dC).poly(dI-dC) in B were very small.

200 μ g protein (figure 3.9 B). However, unlike the -668 to +50 bp fragment, binding was abolished at all concentrations in the presence of poly (dI-dC).poly (dI-dC), indicating that the binding obtained with this DNA fragment was non-specific and displaced by poly (dI-dC).poly(dI-dC).

As additional controls, a 470 bp fragment of cDNA covering the PTH coding region and BSA, which would not be expected to bind, were used. No binding was observed in incubations with receptor and coding region compared to the -668 to +50 bp fragment (figure 3.10 A). At 100 μ g protein, radioactivity retained on the filter with -668 to +50 bp fragment was $288 \pm 63\%$ of control compared to $109 \pm 35\%$ of control with the coding region. This suggests that VDR were binding specifically to the sequences within the region -668 to +50 bp. Likewise, no significant binding was seen when BSA was substituted for receptors (figure 3.10 B).

To determine whether removal of the region -100 to +50 bp from the -668 to +50 bp fragment had any significant effect on binding, these sequences were deleted from the -668 to +50 bp fragment by digestion with Sau3A. The receptors bound to the -668 to -100 bp fragment to the same extent as the -668 to +50 bp fragment with no significant difference between fragments (figure 3.11). At 200 μ g protein, radioactivity retained on the filter was $466 \pm 20\%$ and $482 \pm 133\%$ of control. Thus, removal of the region from -100 to +50 bp did not significantly affect binding, indicating that these sequences are not involved in binding VDR. From these results, the binding site for receptors for $1,25(\text{OH})_2\text{D}_3$ was localised within the region -668 to -100 bp upstream of the initiation site.

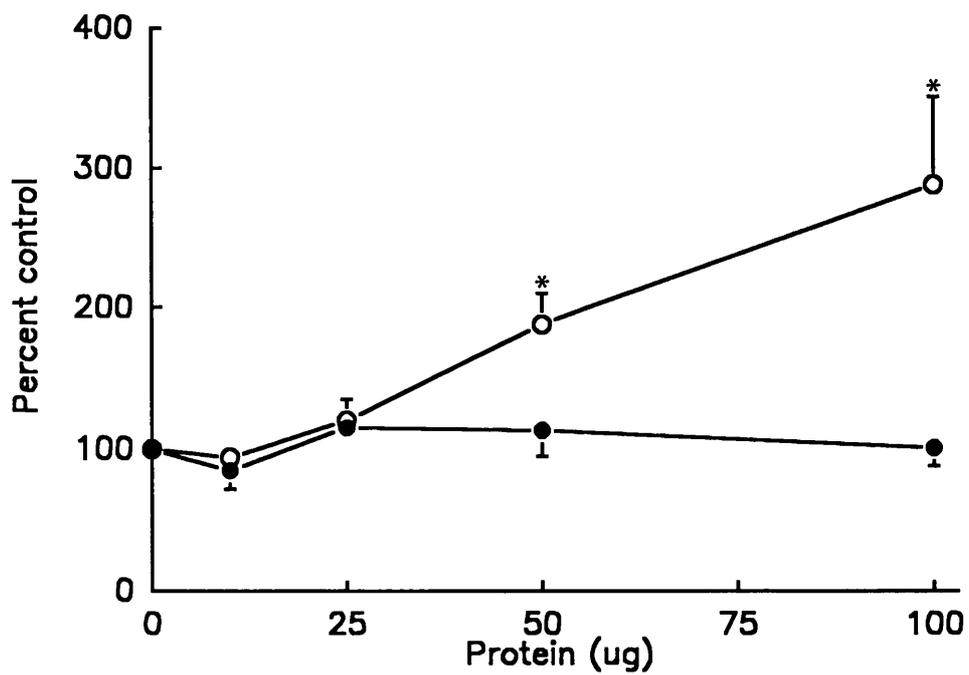
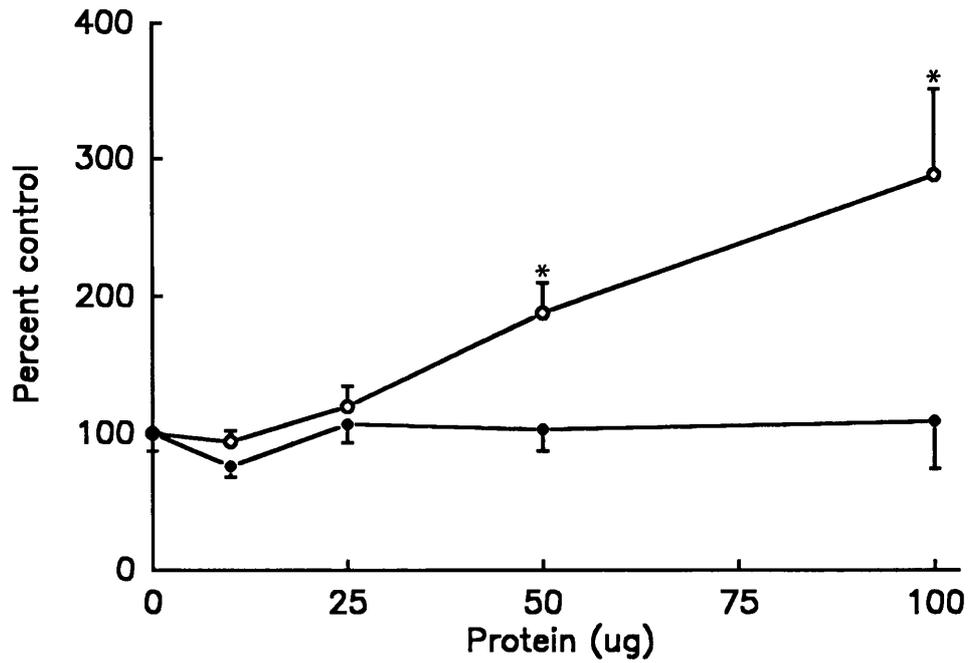


Figure 3.10 Binding of the -668 to +50 bp fragment (o---o) and PTH coding region (•---•) to VDR (A) and binding of -668 to +50 bp fragment (o---o) and BSA (•---•) to VDR (B) in filter binding assays. Results are presented as mean \pm SD with * $p < 0.01$.

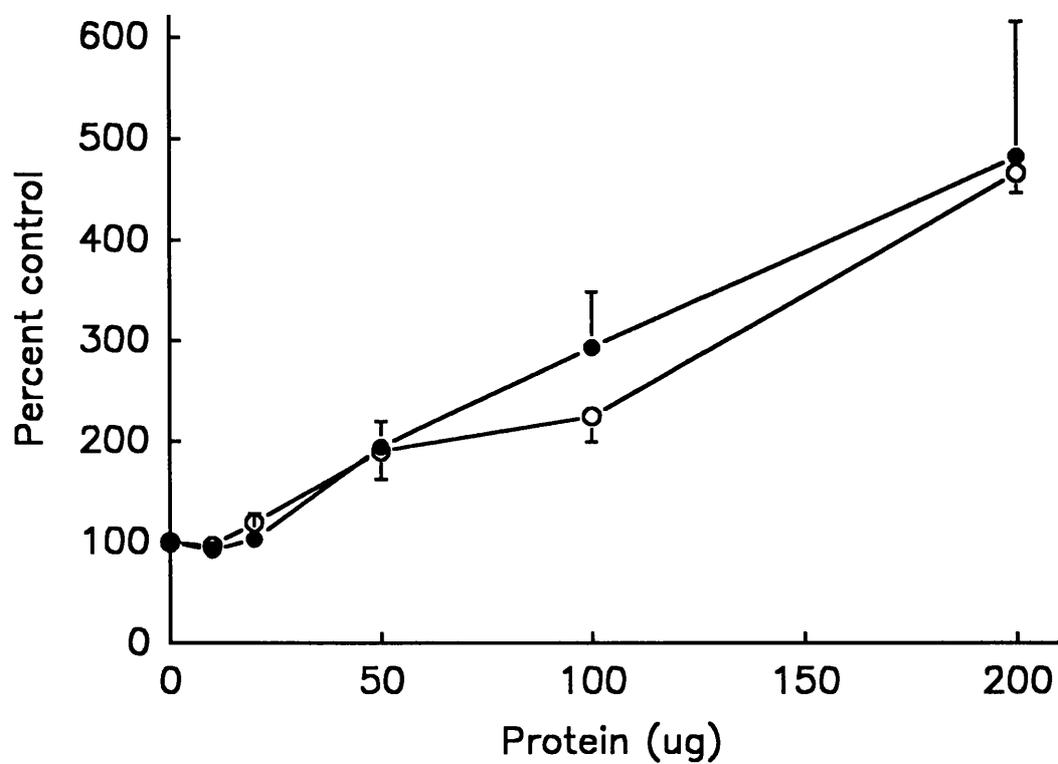


Figure 3.11 shows no significant difference in binding was obtained when the -668 to +50 bp fragment (•---•) and the -668 to -100 bp fragment (○---○) were incubated with receptor protein in filter binding assays. Results are expressed as mean \pm SD.

PART II

Further definition of VDRE

To further define the binding site(s) for VDR, Southwestern assays (immobilised receptor assays) were used in which smaller DNA fragments generated from the -668 to +50 bp fragment were incubated with VDR.

METHODS

DNA fragments

The -668 to +50 bp fragment contains restriction sites for *Hinf*I as shown in figure 3.12 A and this region was digested with *Hinf*I (18 U/ μ g) in buffer B at 37° C for 16 hours. This yielded three fragments of approximately 400, 225 and 100 bp spanning the regions -485 to -50, -668 to -485 and -50 to +50 bp, respectively (figure 3.12 B).

The -668 to +50 bp, -668 to -100, -485 to -50 and -668 to -485 bp fragments and the *Ssp*I-generated fragments described below were dephosphorylated and endlabeled with T4 polynucleotide kinase as described in Appendix I and used in further binding studies of VDR.

Optimisation of partial digestion of the -668 to +50 bp fragment with *Ssp*I

Smaller DNA fragments were generated by digestion of the -668 to +50 bp fragment with *Ssp*I, the presence of which are shown in figure 3.13. Digestion was optimised to yield partially digested fragments, since complete

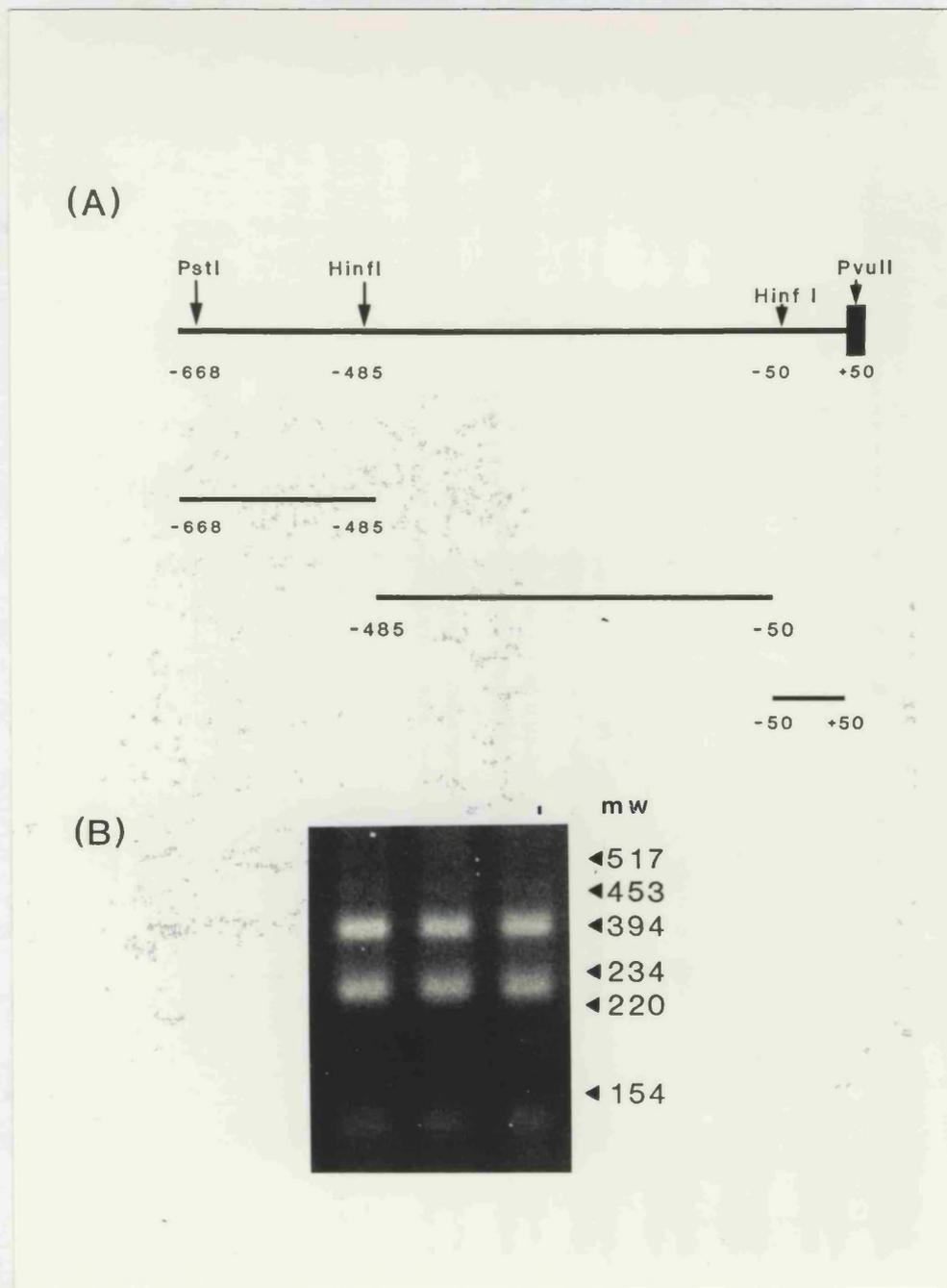


Figure 3.12. The -668 to +50 bp fragment contains restriction sites for HinfI as shown in A and digestion of this region yielded fragments spanning the regions -668 to -485, -485 to -50, and -50 to +50 bp (B). Molecular size markers are shown on the right.

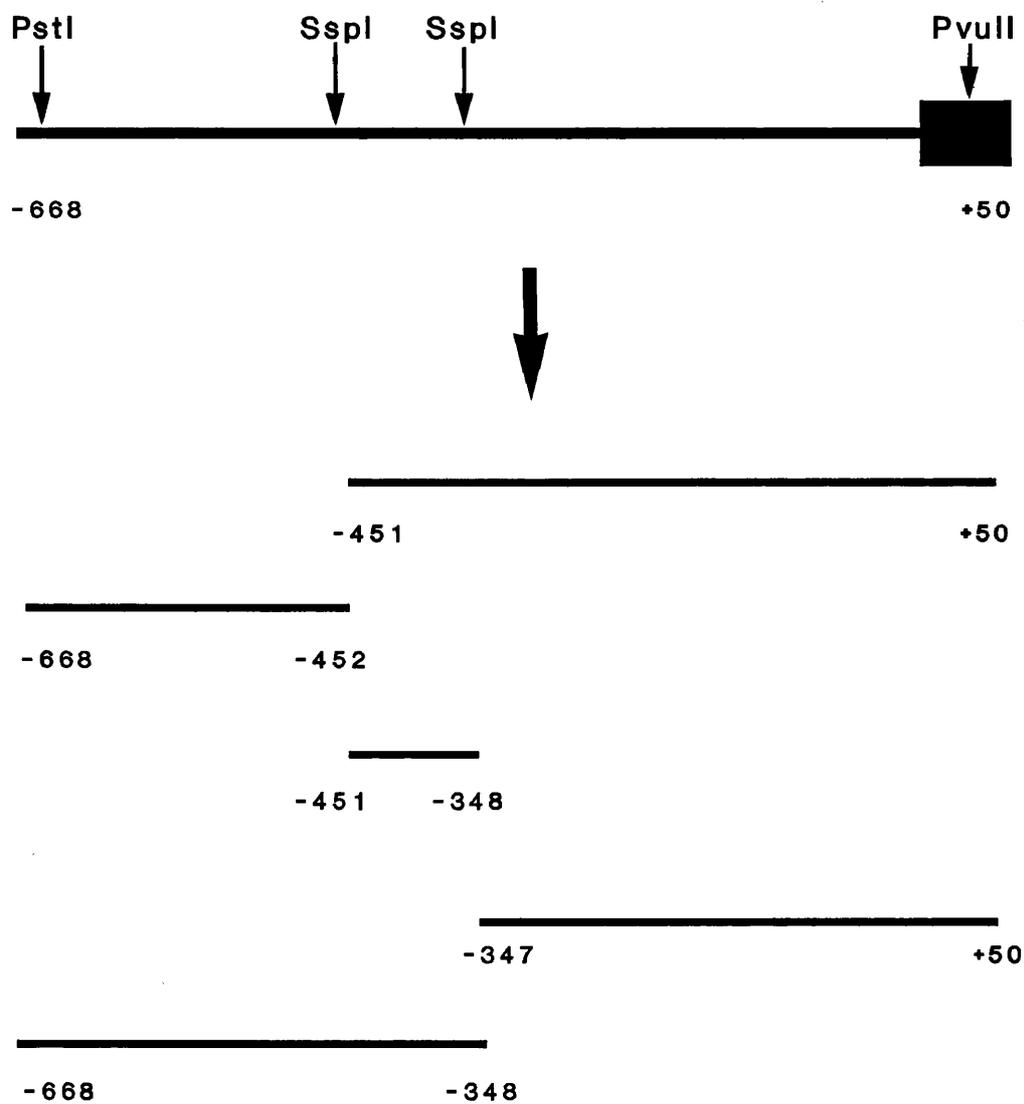


Figure 3.13. Restriction sites for SspI within the -668 to +50 bp fragment are shown. Five fragments are expected: -451 to +50, -347 to +50, -668 to -348, -668 to -452 and -451 to -348 bp.

digestion yielded a large number of small fragments. Digestion was initially optimised for enzyme concentration, in which the -668 to +50 bp fragment (2.5 μg) was digested in buffer H containing 5 mM spermidine at 37°C using 3.6, 7.2 and 16.2 Units Ssp I/ μg DNA for 4 hours at 37°C. In addition, digestion was optimised for 2, 4, 6, and 16 hours.

Digestion with 3.6 and 7.2 Units of SspI/ μg DNA (figure 3.14 A, lanes 1 and 2) yielded five fragments of approximately 500, 450, 350 (faint), 250 and 150 bp, whilst digestion with 16.2 Units of SspI/ μg gave six fragments (lane 3), four of which are the same as in lanes 1 and 2, in addition to fragments of 400 and 100 bp (faint band). Moreover, this did not give a 350 bp fragment seen in lanes 1 and 2 which was expected from the sequence. Optimisation of digestion for 2, 4, 6 and 16 hours with 3.6 Units of Ssp I/ μg (figure 3.14 B) yielded five fragments of approximately 500, 450, 350 (very faint), 250 and 150 bp. Since there was no difference in fragments observed, subsequent digestions were performed for 4 hours at 37°C for convenience and with 3.6 Units of SspI/ μg DNA. These fragments were purified from agarose gels using GeneClean or Mermaid (as described in Appendix I) prior to ligation and sequencing as described later.

Southwestern assay

Following SDS-PAGE and electroblotting of partially purified receptors and parathyroid cell extract as described in methods, proteins immobilised on nitrocellulose filters were incubated in 10 mM Tris-HCl (pH 8.0) containing 4 M urea, 150 mM KCl, 1 mM EDTA and 0.1 mM DTT for 3 hours at 20°C.

Filters were probed in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 0.25% (w/v) DSA, 0.025% (w/v) Ficoll, 0.02% (w/v) Triton X-100 and 100 µg/ml salmon sperm DNA for 1 hour at 20°C

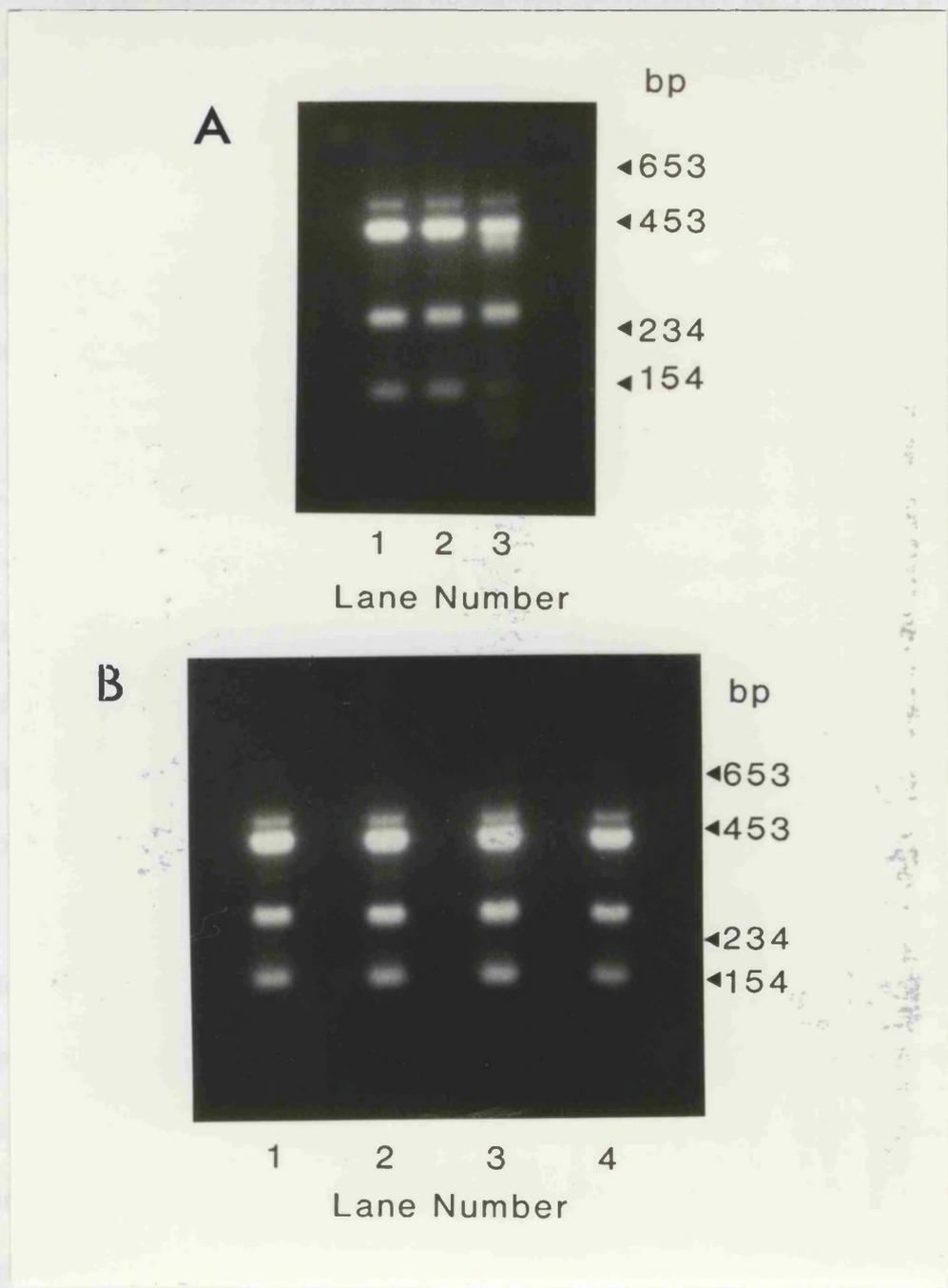


Figure 3.14 (A): Digestion of the -668 to +50 bp fragment with 3.6 (lane 1), 7.2 (lane 2) and 16.2 Units (lane 3) of *SspI*/µg DNA and (B) shows digestion with 16.2 Units of *Ssp I*/µg DNA for 2 (lane 1), 4 (lane 2), 6 (lane 3) and 16 (lane 4) hours. Fragments were resolved by agarose gel electrophoresis. Molecular weights are indicated on the right.

Filters were prehybridised in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM KCl, 0.02% (w/v) BSA, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone and 100 μ g/ml salmon sperm DNA for 1 hour at 20°C before incubation in the same buffer containing 32 P-DNA fragments (10⁵ cpm/ml) radiolabelled with T4 polynucleotide kinase as described in Appendix I for 1 hour at 20°C. Filters were washed in 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 1 mM EDTA at 20°C and the DNA-binding proteins were visualised by autoradiography at -70°C.

Cloning of SspI-generated fragments into pBluescript plasmid

Due to the problems of identification of the SspI-generated fragments described above by restriction enzyme digestions, these fragments were cloned into the plasmid vector, pBluescript II SK (+) (Stratagene, Cambridge, U.K.) (plasmid map in Appendix III) before being sequenced using Sanger's method of dideoxy-mediated chain termination (as described later).

SmaI (11 Units) was used to linearise 5 μ g pBluescript in buffer B at 37°C for 16 hours. At the end of the incubation, proteins were removed by phenol:chloroform extraction and DNA was concentrated by precipitation in alcohol at -20°C. The linearised plasmid was dephosphorylated using calf intestinal alkaline phosphatase (CIP) as described in Appendix I to prevent reannealing .

As described in Appendix I, DNA fragments -668 to -348 and -668 to -452 bp were blunt-ended (see filling recessed 3'ends) before ligation into the SmaI site of dephosphorylated pBluescript plasmid catalysed by the enzyme T4

DNA ligase and prior to transformation of XLI-blue competent cells. In general, 6-10 colonies were picked and restriction enzyme digestions using a combination of sites in pBluescript and PTH fragments were carried out in order to linearise the construct and digest the insert DNA. The expected sizes quoted for all digestions were determined from the published sequence (Weaver *et al.* 1984) whilst those obtained were determined from agarose gels by plotting the molecular weight markers.

a) Fragment -451 to +50 bp

Ten clones containing the -451 to +50 bp fragment in pBluescript plasmid were digested with PstI (figure 3.15 A) which linearises the construct and HindIII to prove that the insert was present (figure 3.15 B). Lane 1 represent linearised pBluescript plasmid in both figures. In most cases, digestion with PstI (A) yielded a fragment at 3500 bp (lanes 2-5, 7-11), but the clone in lane 6 gave a band at about 4500 bp and therefore was not correct. Digestion with HindIII (B) which is present in the PTH fragment, gave a fragment of 420 bp (see lanes 3 and 5 figure 3.15 B), indicating the presence of the -451 to +50 bp fragment in the plasmid and, in addition to a fragment of 3300 bp. A fragment of 420 bp was also observed in lane 6, however, since additional fragments were seen, this clone was discarded. Thus, lanes 3 and 5 were subsequently sequenced.

b) Fragment -347 to +50 bp

Six clones containing the fragment -347 to +50 bp in pBluescript plasmid

were each digested with EcoRI (lanes 2-7, figure 3.15) and HindIII (lanes 8-11). Lane 1 represents linearised pBluescript plasmid used as a control. A useful clone would be one which when digested with EcoRI gave a fragment

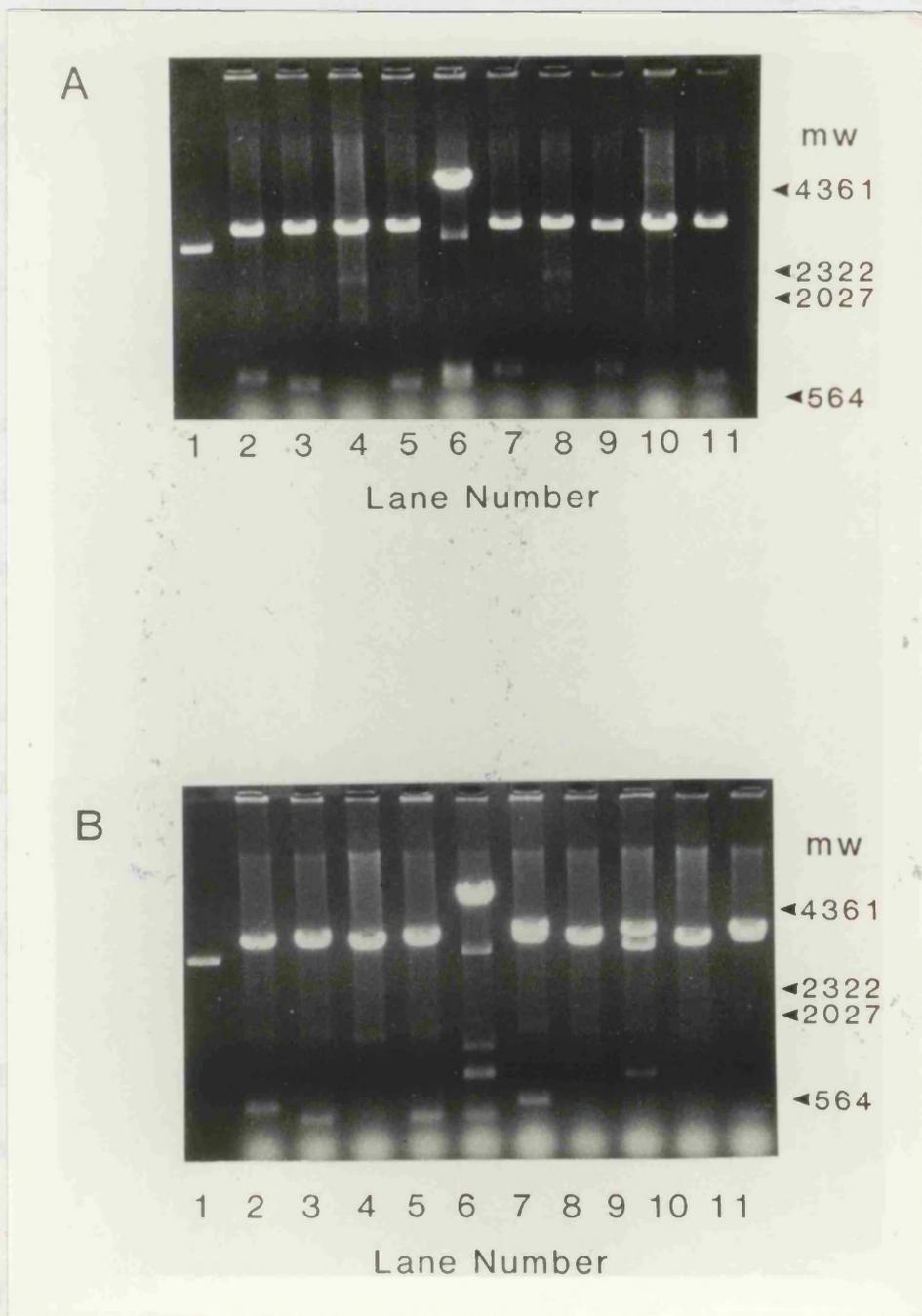


Figure 3.15. Digestion of ten clones containing the -451 to +50 bp fragment in pBluescript plasmid with Pst I (A) and Hind III (B). Lane 1 represents linearised pBluescript plasmid in both panels.

were each digested with EcoRI (lanes 2-7, figure 3.16) and HindIII (lanes 8-13). Lane 1 represents linearised pBluescript plasmid used as a control. A useful clone would be one which when linearised with EcoRI gave a fragment of 3400 bp and two fragments of 2900 and 680 bp with HindIII. The only clone which met both these requirements was that seen in lanes 2 and 8.

c) Fragment -668 to -348 bp

Nine clones containing the fragment -668 to -348 bp in pBluescript plasmid were digested with PstI which linearises the construct and PstI + XbaI which removes the insert DNA. The most informative test is the double digestion using PstI + XbaI which gives an insert of 320 bp (figure 3.17 A) in addition to a 3000 bp fragment. These clones are seen in lanes 5, 6 and 8. Furthermore, these clones when digested with PstI (figure 3.17 B) gave a 3260 bp fragment and lane 8 was subsequently sequenced.

d) Fragment -668 to -452 bp

Similarly, six clones containing the PTH fragment -668 to -452 bp in pBluescript plasmid were each digested with PstI (figure 3.18, lanes 2-7) and PstI + XbaI (lanes 8-13). Lane 1 represents linearised pBluescript plasmid. A useful clone would be one which when linearised with PstI gave a fragment of 3200 bp and this was observed in lanes 4, 6, and 7. Once again, the choice of test is the double digestion using PstI + XbaI which should give an insert of 230 bp and this was seen lanes 10, 12 and 13. A number of smaller fragments resulted after digestion with PstI and PstI + XbaI, however,

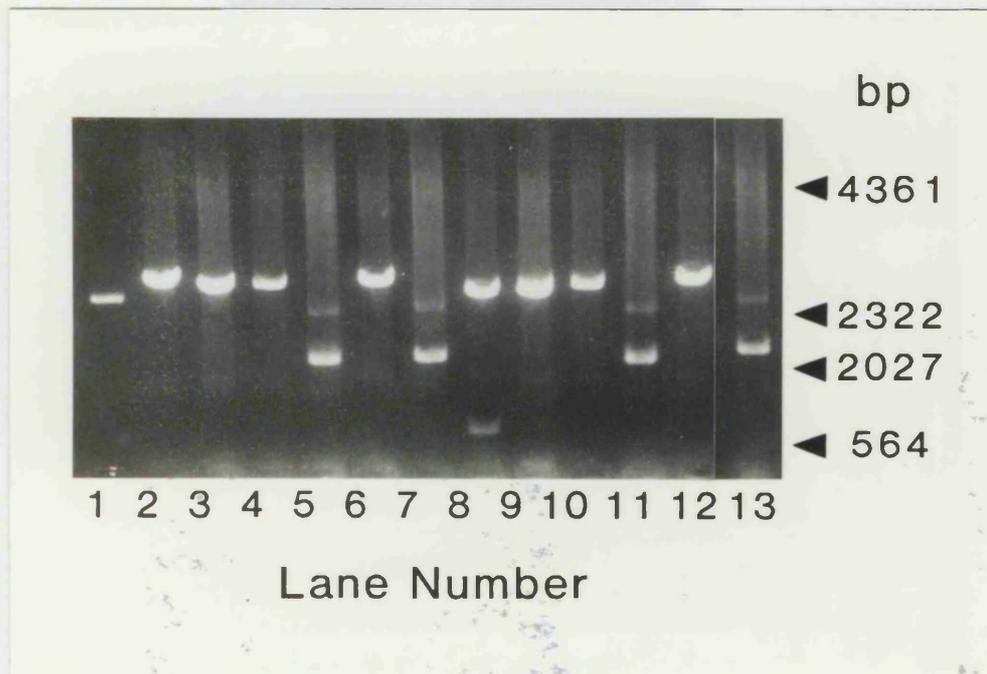
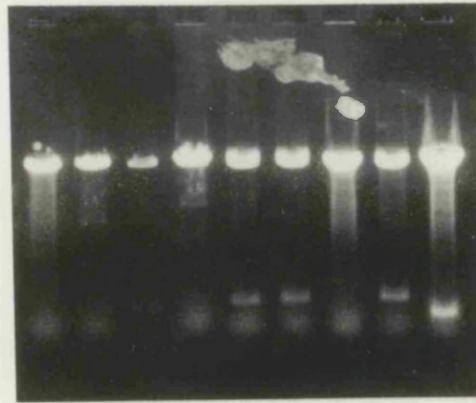


Figure 3.16. Digestion of 6 clones containing the -347 to +50 bp fragment in pBluescript plasmid with EcoRI (lanes 2-7) and with HindIII (lanes 8-13). Lane 1 represents linearised pBluescript plasmid.

A

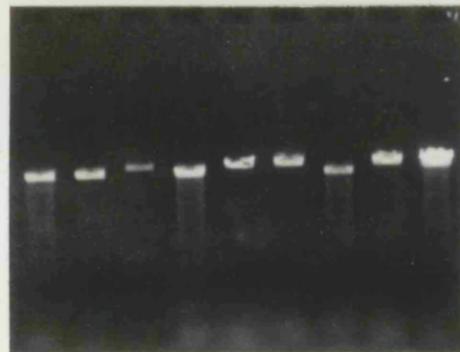


mw
◀ 4361
◀ 2322
◀ 2027
◀ 564

1 2 3 4 5 6 7 8 9

Lane Number

B



mw
◀ 4361
◀ 2322
◀ 2027
◀ 564

1 2 3 4 5 6 7 8 9

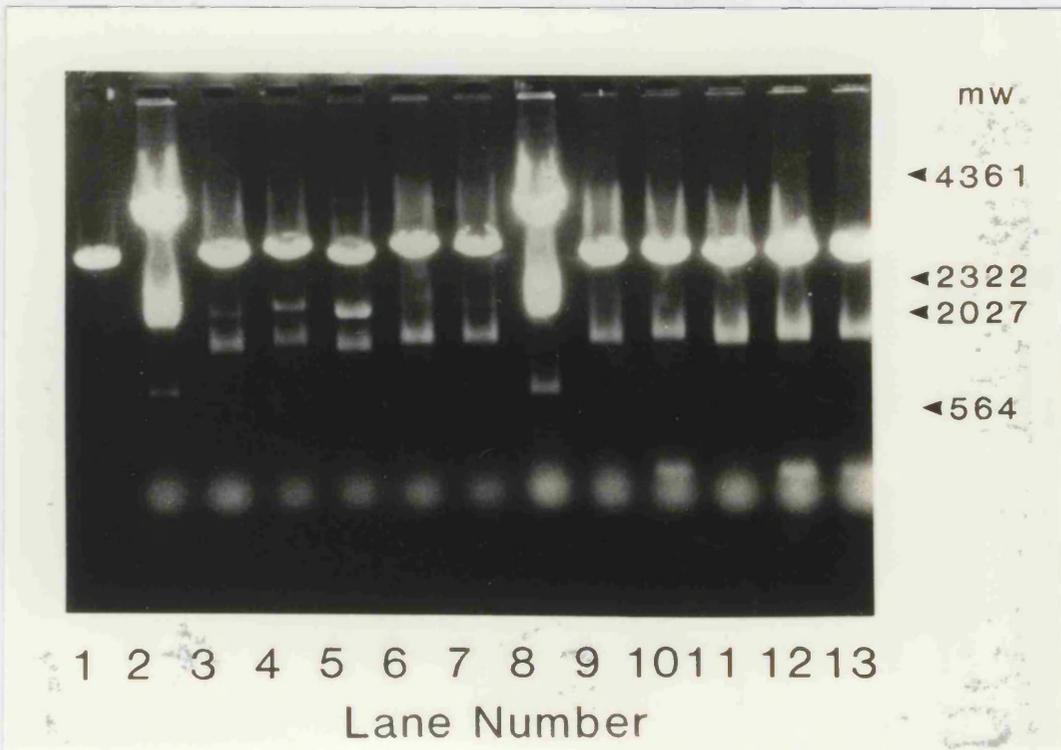
Lane Number

Figure 3.17. Digestion of 9 clones containing the -668 to -348 bp fragment in pBluescript plasmid with PstI (A) and PstI + XbaI (B).

Some of the clones were observed in lane 4 which was subsequently sequenced.

3.19. Fragment -551 to -344 bp

Seven clones containing the -551 to -344 bp fragment in pBluescript plasmid were digested with PvuII and HglI (figure 3.19 A and B, respectively). Useful clones would be ones which gave fragments of 2500 and



Template DNA (2 µg) was denatured with 0.4 M NaOH at 20°C for 10 minutes followed by the addition of 3 M sodium acetate (pH 4.8) (3 µl), distilled water (7 µl) and 100% ethanol (50 µl), mixed and placed on dry ice for 15 minutes. DNA was collected by centrifugation, the pellet dried under vacuum and redissolved in 10 µl distilled water. To the denatured template

Figure 3.18. Digestion of 6 clones containing the -668 to -452 bp fragment in pBluescript plasmid with PstI (2 -7) and PstI + XbaI (8 - 13). Lane 1 represents linearised pBluescript plasmid.

these did not appear after caesium chloride ultracentrifugation. The above criteria were observed in lane 4 which was subsequently sequenced.

e) Fragment -451 to -348 bp

Seven clones containing the -451 to -348 bp fragment in pBluescript plasmid were digested with PvuII and BglI (figure 3.19 A and B, respectively). Useful clones would be ones which gave fragments of 2500 and 600 bp with PvuII (A) and fragments of 1850 and 1300 bp with BglI (B). The only clone to meet both these criteria was observed in lane 6 (A and B) and this clone was subsequently sequenced.

Sequencing reactions

The unlinearised double-stranded plasmids containing the DNA fragments were subsequently sequenced using bacteriophage T7 DNA polymerase by Sanger's method of dideoxy-mediated chain termination. The reactions were performed using a T7 sequencing kit (Pharmacia LKB Biotechnology Ltd, Uppsala, Sweden).

Template DNA (2 μg) was denatured with 0.4 M NaOH at 20°C for 10 minutes followed by the addition of 3 M sodium acetate (pH 4.8) (3 μl), distilled water (7 μl) and 100% ethanol (60 μl), mixed and placed on dry ice for 15 minutes. DNA was collected by centrifugation, the pellet dried under vacuum and redissolved in 10 μl distilled water. To the denatured template DNA (10 μl), 2 μl annealing buffer (1 M Tris-HCl (pH 7.6) containing 100 mM MgCl₂ and 160 mM DTT) and 2 μl primer solution (0.8 μM) was added

and incubated at 37°C for 30 minutes. Following this, the reaction mixture was incubated at 20°C for 15 minutes and then centrifuged briefly. The

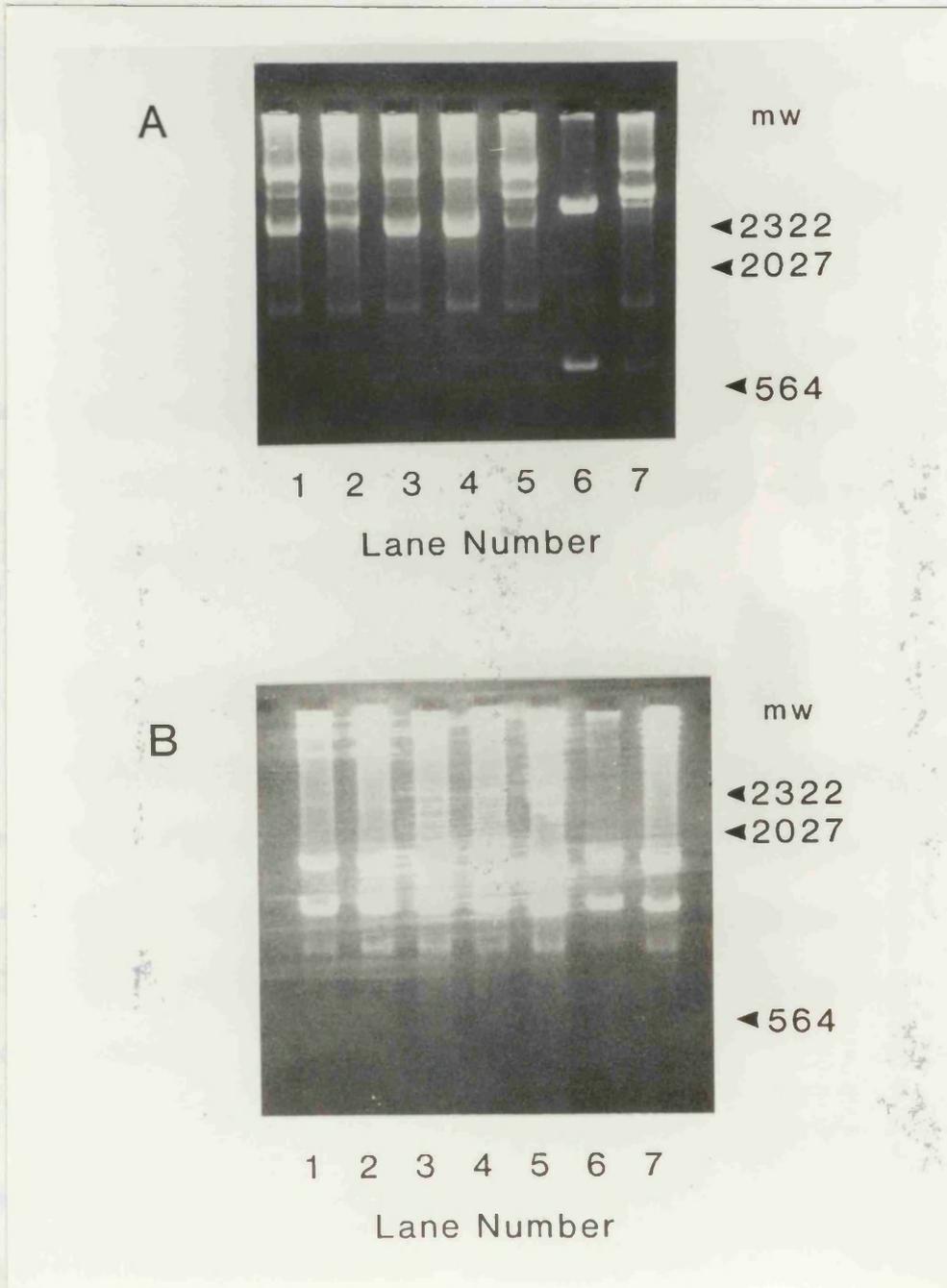


Figure 3.19. Digestion of seven clones containing the -451 to -348 bp fragment in pBluescript plasmid with PvuII (A) and BglI (B).

Labelling reaction mixture (4.5 µl) was added to each of the four pro-

and incubated at 37°C for 20 minutes. Following this, the reaction mixture was incubated at 20°C for 10 minutes and then centrifuged briefly. The primer used was a single-stranded universal primer consisting of 17 oligonucleotides which anneals to its complementary sequence in pBluescript just outside the multiple cloning site:



An enzyme premix was prepared by depositing the following reagents on the side of a microfuge tube on ice and mixed briefly by centrifugation:

Water	1 μ l
Labelling mix-dATP(dCTP, dGTP and dTTP)	3 μ l
T7 DNA polymerase (1.5 Units/ μ l)	2 μ l
[α - ³⁵ S]dATP α S (1 μ l = 10 μ Ci)	1 μ l
Total volume =	7 μ l

Enzyme premix (6 μ l) was added to the annealed template and primer, contents mixed gently and incubated at 20°C for 5 minutes. Whilst this incubation was in progress, 2.5 μ l of each of the sequencing mixes i.e. 'A', 'C', 'G' and 'T' were deposited on the side of the microfuge tube and incubated for 37° C for approximately two minutes.

Each sequencing mix contains one dideoxy nucleotide in addition to three deoxy nucleotides as follows:

'A' contains ddATP in solution with dATP, dCTP, dGTP and dTTP

'C' contains ddCTP in solution with dATP, dCTP, dGTP and dTTP

'G' contains ddGTP in solution with dATP, dCTP, dGTP and dTTP

'T' contains ddTTP in solution with dATP, dCTP, dGTP and dTTP

Labelling reaction mixture (4.5 μ l) was added to each of the four pre-

warmed sequencing mixes on the side of the microfuge tube. The reaction was started by brief centrifugation, incubated at 37°C for 5 minutes and terminated by the addition of 5 μ l deionised formamide solution containing EDTA, xylene cyanol and bromophenol blue to the side of the microfuge tube.

Each reaction (3 μ l) was heated to 75°C for 2 minutes and 2 μ l was loaded immediately onto a pre-electrophoresed 6% or 8 % denaturing polyacrylamide gel (Sequagel, National Diagnostics, USA). The dimensions of the gel plates used were 20 X 40 cm and 0.4 mm thick. Electrophoresis was performed at constant voltage of 1200 V in 89 mM Tris, 89 mM boric acid and 1 mM EDTA (pH 8.3) buffer. The gel was fixed in 10 % methanol/10 % acetic acid in water for 20 minutes at 20°C, transferred onto Whatman filter paper and dried at 65°C for 50-60 minutes. The dry gel was exposed to X-ray film for 24 hours at 20°C.

RESULTS

Sequencing of SspI-generated fragments

The sequencing of SspI-generated fragments confirmed the sizes and identity of these fragments. Figures 3.20 - 3.24 show autoradiographs following sequencing of the -451 to +50 bp, -347 to +50 bp, -668 to -348 bp, -668 to -452 bp and -451 to -348 bp regions in pBluescript plasmid. The SspI fragments were inserted into the SmaI site of pBluescript (GGGCCCC) as indicated, for example, in the -451 to -348 bp fragment. In addition, a short stretch of sequence from each of the fragments is shown. The sequences compared well with the published sequence of the bovine PTH gene (Weaver *et al.* 1984).

The region from -668 to -510 bp had not been sequenced previously thus two clones containing the region -668 to -452 bp in the forward, that is, 5' to 3' and back, that is, 3' to 5' orientation in pBluescript SK (+) plasmid were sequenced and the sequence from -668 to -500 is shown in figure 3.25. The region from -668 to -510 bp was analysed for restriction sites for the enzymes used for digestions in this study as this sequence is not published. The programme Microgenie was used and the analysis is shown in Appendix III.



Figure 3.20. Autoradiograph following sequencing of the -451 to +50 bp region of the bovine PTH promoter in pBluescript plasmid. The *Sma*I site of pBluescript where the PTH fragment was inserted is indicated by CCCG and the PTH fragment from -451 to -437 bp is shown.

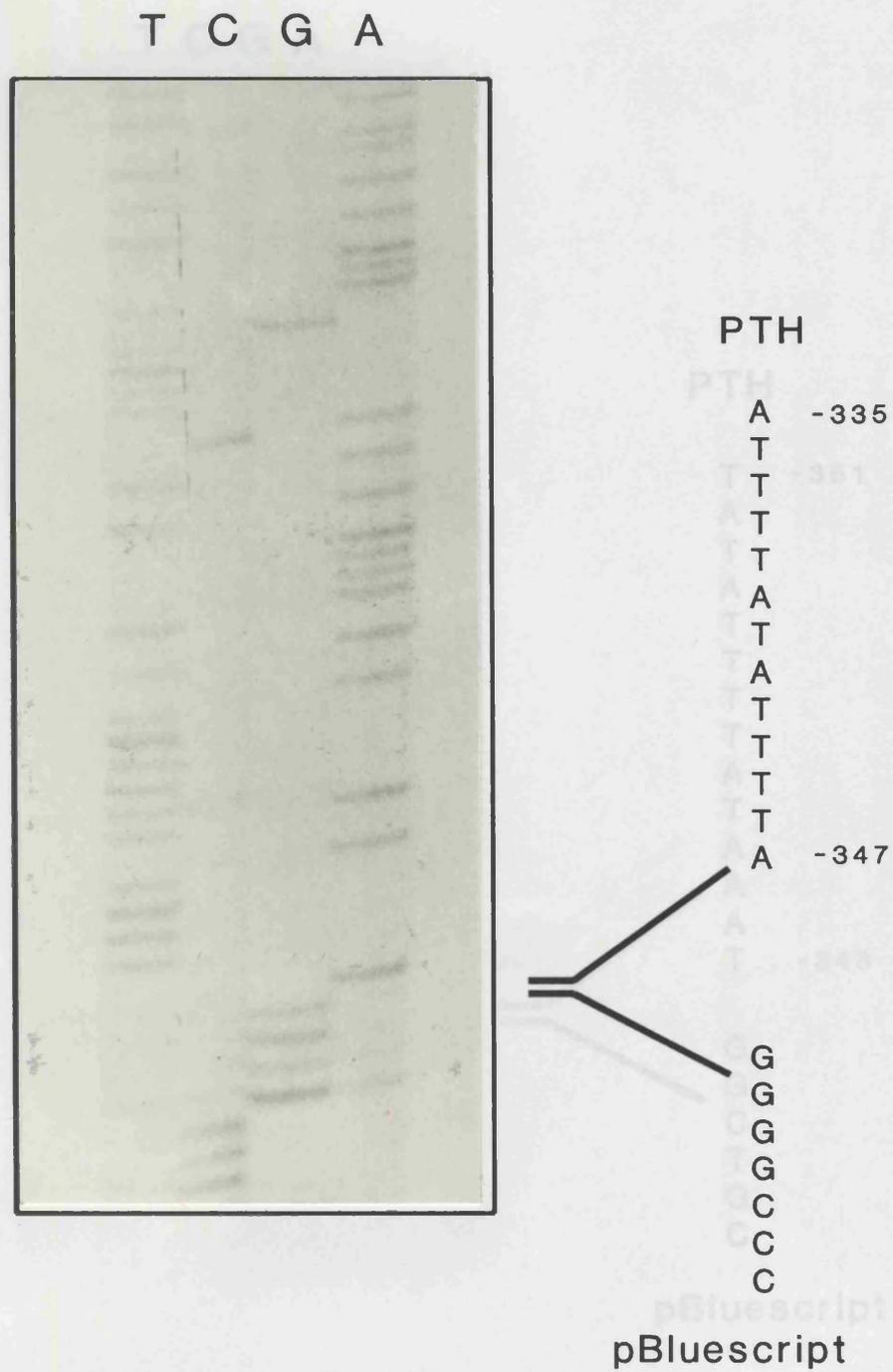


Figure 3.21. Autoradiograph following sequencing of the -347 to +50 bp region of the bovine PTH promoter in pBluescript plasmid. The *Sma*I site of pBluescript where the PTH fragment was inserted is indicated by CCCGGGG and the PTH fragment from -347 to -335 bp is shown.

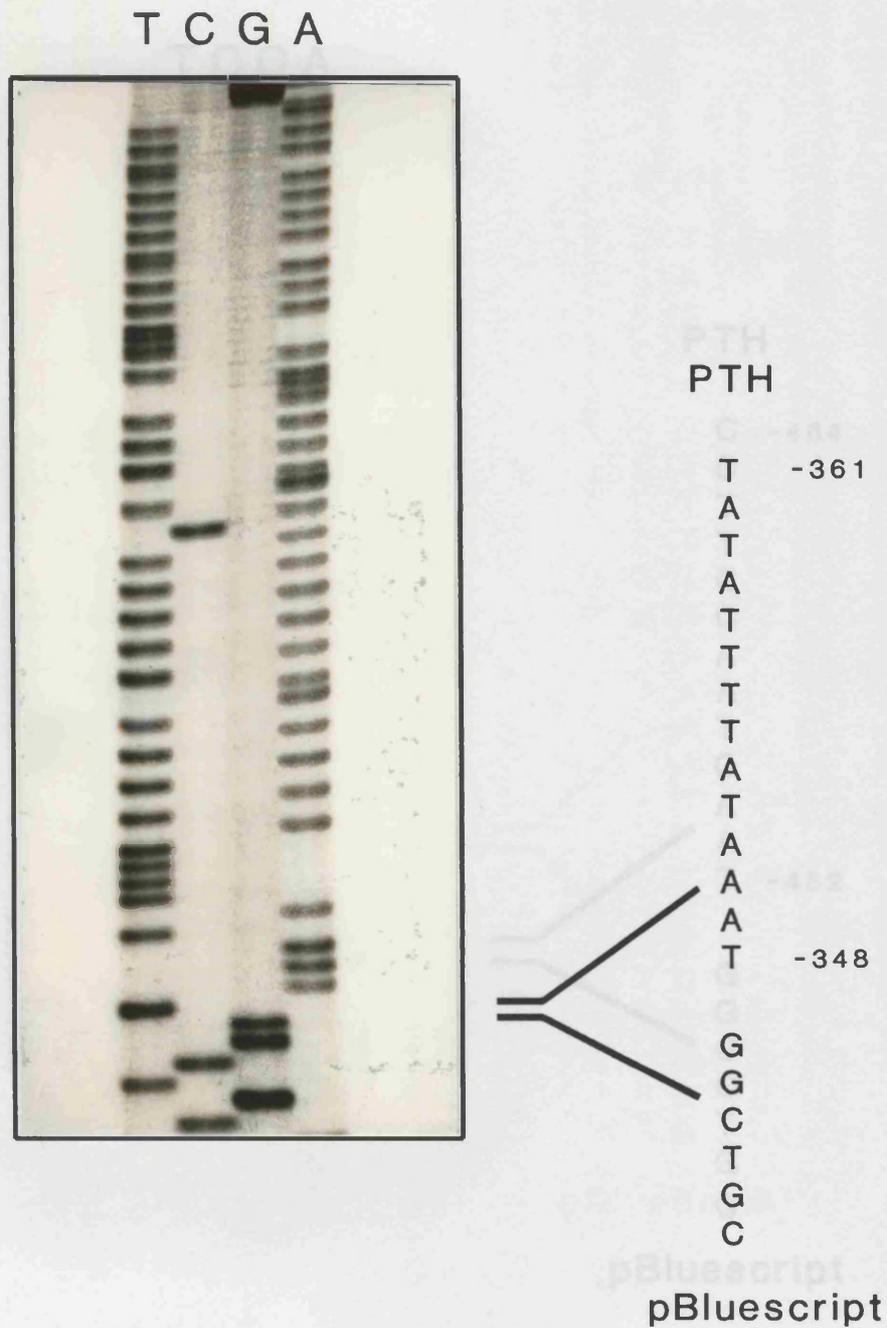
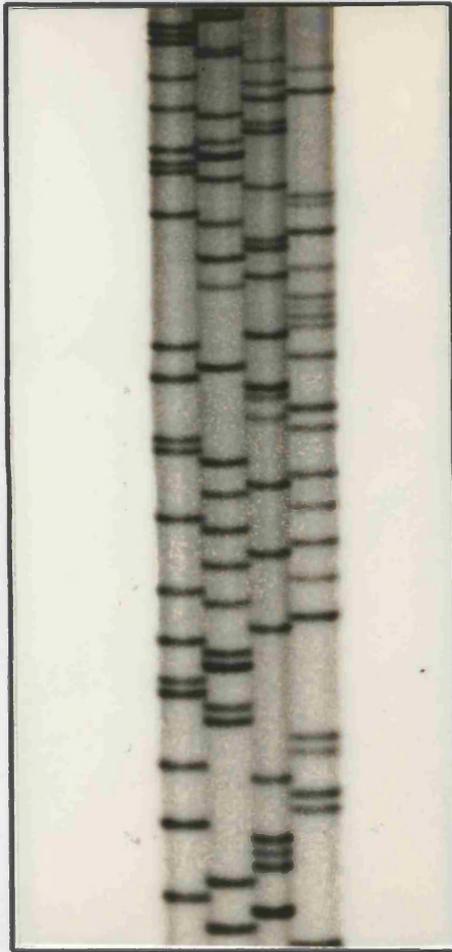


Figure 3.22. Autoradiograph following sequencing of the -668 to -348 bp region of the bovine PTH promoter in pBluescript plasmid. The *Sma*I site of pBluescript where the PTH fragment was inserted is indicated by CGTCCG and the PTH fragment from -348 to -361 bp is shown.

T C G A



PTH

PTH

C -464

C

T

T

C

C

A

A

A

A

A

A

T -452

C

C

G

G

G

C

T

G

C

pBluescript

Figure 3.23. Autoradiograph following sequencing of the -668 to -452 bp region of the bovine PTH promoter in pBluescript plasmid. The *Sma*I site of pBluescript where the PTH fragment was inserted is indicated by GGGCTGC and the PTH fragment from -452 to -464 bp is shown.



Figure 3.24. Autoradiograph following sequencing of the -451 to -348 bp region of the bovine PTH promoter in pBluescript plasmid. The SmaI site of pBluescript where the PTH fragment was inserted is indicated by GGGCCCC and a small region of the PTH fragment from -348 to -363 bp is shown.

Southwestern assays

Partially purified VDR and parathyroid cytosol were separated by SDS-PAGE and transferred to nitrocellulose filters as described previously and incubated with radiolabelled -668 to +50 bp fragment for the Southwestern assay. Equivalent filters were incubated with monoclonal antibodies to VDR in Western blots as shown previously. Figure 3.26 shows autoradiographs of filters containing partially purified receptors for $1,25(\text{OH})_2\text{D}_3$ (lane 1) and parathyroid cytosol (lane 2) reacted with monoclonal antibody (A) and equivalent filters with radiolabelled -668 to +50 bp fragment (B lanes 3 and 4). As described previously, a single band at a molecular mass of approximately 50 000 Da was present in immunoblots of partially purified receptors and parathyroid cytosol. This band was coincident with the band obtained following incubation of equivalent filters with the -668 to +50 bp fragment (lanes 3 and 4) suggesting that the DNA-binding protein was VDR. No smaller bands that would indicate degradation of receptor were present. It should be noted that this is a qualitative rather than a quantitative figure since the autoradiographs have been exposed for different time periods. Standard protein molecular weight markers are indicated on the right.

To further localise the binding sites for VDR within the -668 to +50 bp region, this fragment was digested with *Sau3A* and *HinfI* as described in methods and the fragments generated were used in Southwestern assays. Incubation of immobilised receptors with radiolabelled -668 to +50 bp (lane 1), -668 to -100 bp (lane 2) and -485 to -50 bp (lane 3) fragments (figure 3.27), gave a single band at a molecular mass of approximately 50 000 Da.

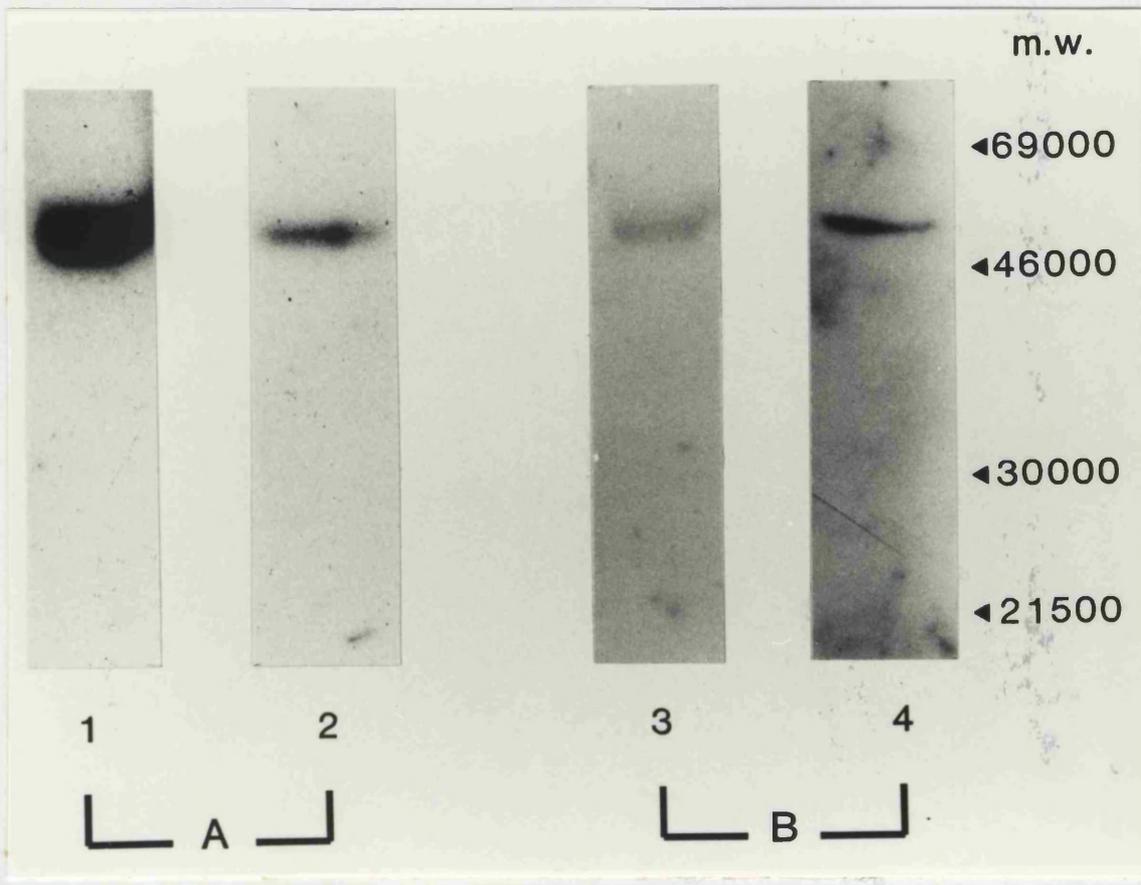
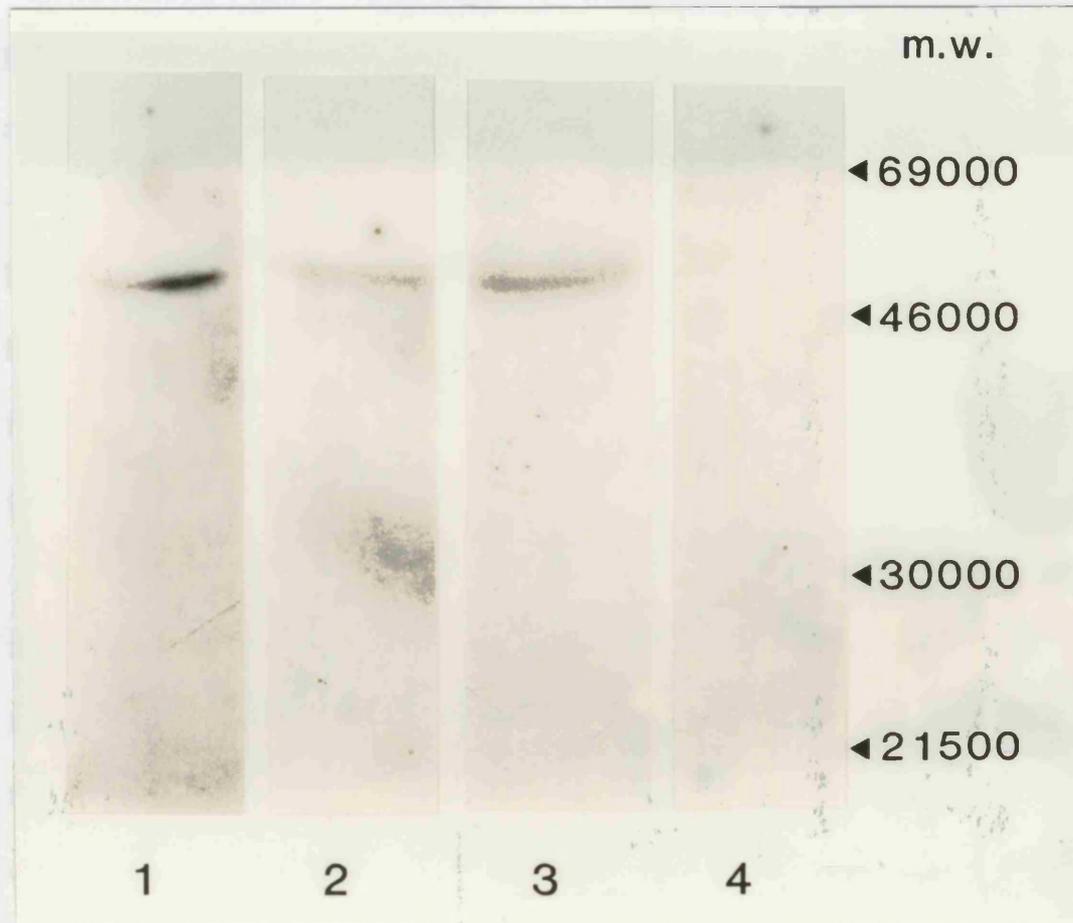


Figure 3.26 shows autoradiographs of reactions of filters containing partially purified receptors (lane 1), parathyroid cytosol (lane 2) with monoclonal antibodies (A) and equivalent filters (B) with the -668 to +50 bp fragment (lanes 3-4). Molecular weight markers are indicated on the right.

As before, this band was coincident with that in immunoblots. In contrast, binding to the -668 to -485 bp fragment (lane 4) was not observed. Thus, the VDR binding site was localized to within -485 to -100 bp upstream of the initiation site.

This site was further defined using the *SapI*-generated DNA fragments and Figure 3.28 shows autoradiographs following incubation of filters containing



Southwestern assays (Part II), the VDR binding domains in the bovine PTH gene were localized to within -485 to -100 bp upstream of the initiation site (Figure 3.28).

Figure 3.27. Autoradiographs following incubation of receptors for $1,25(\text{OH})_2\text{D}_3$ immobilised on nitrocellulose after SDS-PAGE with the -668 to +50 (lane 1), -668 to -100 (lane 2), -485 to -50 (lane 3) and -668 to -485 bp (lane 4) fragments of the bovine PTH gene. Molecular weight markers are indicated on the right.

As before, this band was coincident with that in immunoblots. In contrast, binding to the -668 to -485 bp fragment (lane 4) was not observed. Thus, the VDR binding site was localised to within -485 to -100 bp upstream of the initiation site.

This site was further defined using the *Ssp*I-generated DNA fragments and figure 3.28 shows autoradiographs following incubation of filters containing partially purified VDR with these DNA fragments. A single band was seen again following incubation with -451 to +50 (lane 1) and with the fragment -451 to -348 bp (lane 2). In contrast, the sequence -347 to +50 bp did not bind (lane 3). Binding occurred with the fragment -668 to -452 bp (lane 4), however, the presence of the sequence from -451 to -348 bp (as in the fragment -668 to -348 bp) appeared to inhibit binding to the upstream region (lane 5).

Using Southwestern assays, specific binding was localised within two DNA fragments spanning the regions -451 to -348 bp and -668 to -452 bp. Incubation of equivalent filters containing VDR with monoclonal antibodies to the receptors indicated that the DNA binding protein was VDR. Thus, combining data from filter binding assays (Part I) with the results from Southwestern assays (Part II), two VDR binding domains in the bovine PTH gene were localised to within -485 to -348 bp upstream of the initiation site (figure 3.29).

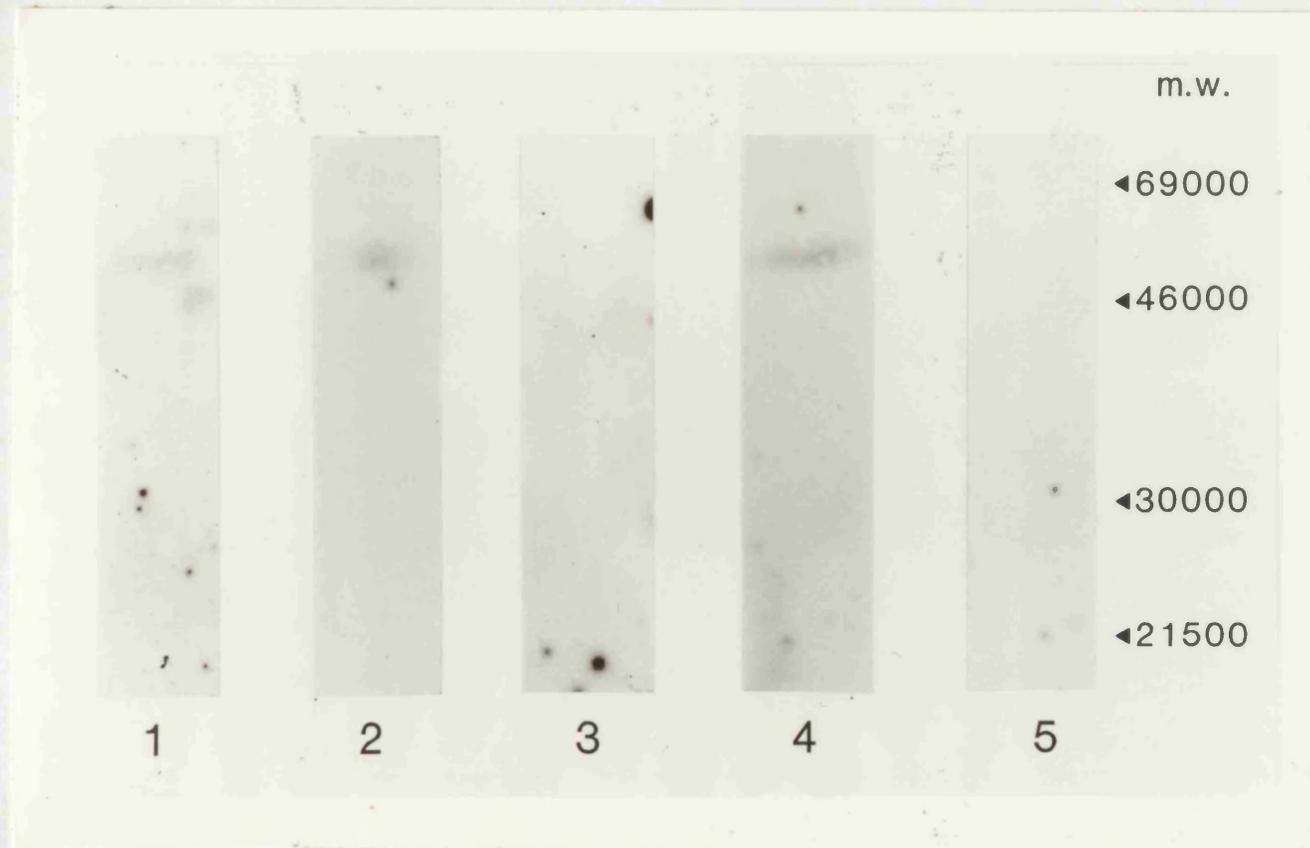


Figure 3.28. Autoradiographs following incubation of receptors for $1,25(\text{OH})_2\text{D}_3$ immobilised on nitrocellulose after SDS-PAGE with -451 to +50 (lane 1), -451 to -348 (lane 2), -347 to +50 (lane 3), -668 to -347 (lane 4) and -668 to -452 bp (lane 5) SspI-generated fragments.

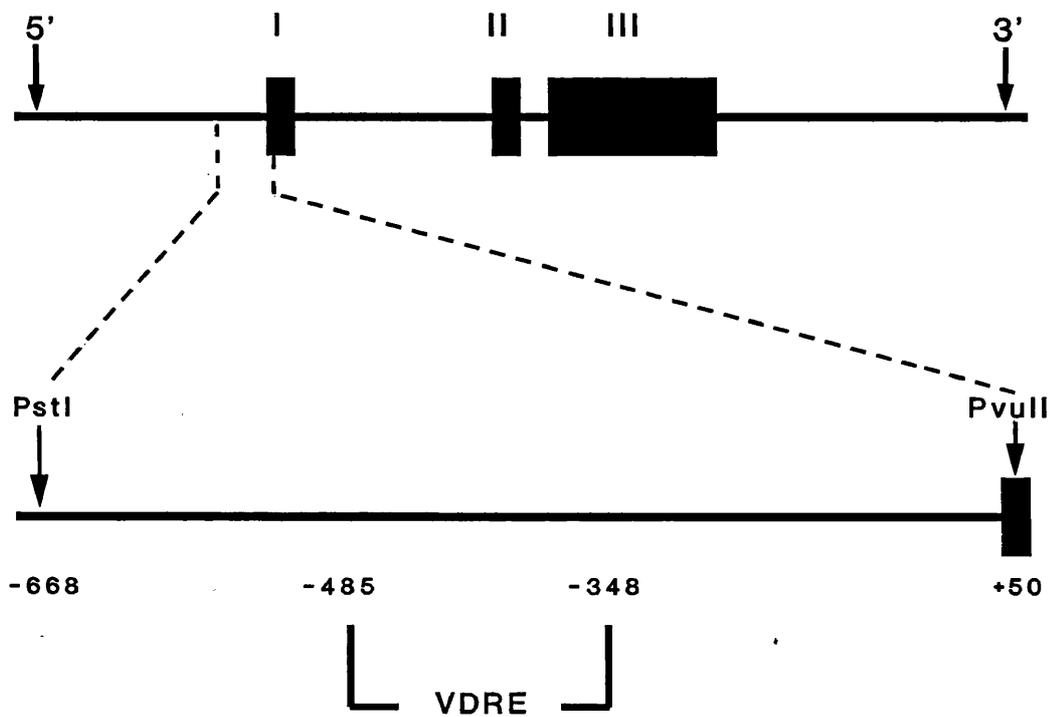


Figure 3.29 shows the region in the bovine PTH gene involved in binding vitamin D₃ receptors located between -485 and -348 bp upstream of the initiation site.

PART III

Characterisation of protein-DNA interactions

To study protein-DNA interactions further, gel mobility shift assays were used, in which SspI-generated fragments were incubated with and without parathyroid cell extract.

METHODS

Generation of DNA fragments

To increase the yield of DNA fragments for the gel mobility shift assays, the -668 to -452 and -451 to -348 bp SspI-generated fragments in pBluescript plasmid were purified by caesium chloride ultracentrifugation rather than digestion of the -668 to +50 bp region with SspI.

Plasmid pBluescript containing SspI DNA fragments (170 μg) were digested with PstI (1.2 U/ μg) and XbaI (1.2 U/ μg) in buffer H for 16 hours at 37°C. After the termination of the reaction with 0.1 volume DNA loading buffer, DNA fragments were separated on a 0.8% agarose gel containing ethidium bromide (1.25 $\mu\text{g}/\text{ml}$). These fragments were excised out of the gels, purified and endlabelled using T4 polynucleotide kinase as described in Appendix I for use as a probe in gel shift assays.

Optimisation of gel shift assays

Parathyroid cytosol (10, 20 or 40 μg) was incubated with radiolabelled

-451 to -348 bp fragment (7500 cpm/ng) end-labelled with T4 polynucleotide kinase in 5 mM HEPES (pH 7.9) containing 75 mM KCl, 0.125 mM DTT, 0.05 mM EDTA and 10 μ g poly (dI-dC).poly (dI-dC) in a final volume of 50 μ l at 20°C for 30 minutes. BSA instead of parathyroid cytosol was included as a negative control. Reaction mixtures were loaded onto pre-electrophoresed 4% non-denaturing polyacrylamide gels and electrophoresis was performed in TBE at 125 V for 4 hours. The gels were fixed in 10% methanol/10% acetic acid for 20 minutes before being dried at 65°C for 50-60 minutes and exposed to X-ray films at -70°C.

In subsequent assays, endlabelled SspI fragments -451 to -348 bp (7500 cpm/ng) and -668 to -452 bp (7500 cpm/ng) were incubated in the presence or absence of 40 μ g of parathyroid cytosol and 10 μ g poly (dI-dC).poly(dI-dC) as described above.

For competition studies, 1000-fold excess of unlabelled -451 to -348 bp, -668 to -452 bp and -347 to +50 bp fragments were added to the reaction mixture. To confirm that the protein involved in these reactions was VDR, 10 μ g monoclonal antibodies to receptors (9A7) was included. In addition, non-specific monoclonal antibodies to PTH (rat IgG antibodies) were added to the incubation medium as control. 1000-fold excess of each unlabelled oligonucleotide consensus sequence for AP1, AP2, AP3 and SP3 was added to the incubation medium in the presence of -452 to -348 bp and -668 to -452 bp fragments.

RESULTS

Gel mobility shift assays were optimised for parathyroid cytosol using the -451 to -348 bp fragment. In the absence of parathyroid cytosol, a single band of free DNA (\blacktriangleleft) was observed (figure 3.30, lane 1). The addition of increasing concentration of cytosol (lanes 2-4) resulted in a second band of lower mobility (\blacktriangleleft) of protein-DNA complex. However, the substitution of BSA for cytosol did not result in retardation of DNA (lane 5). A strong signal was observed with 40 μ g of parathyroid cytosol and was thus used in subsequent assays.

Specificity of the observed protein-DNA interaction with the -451 to -348 bp DNA fragment was supported by results from competition studies. In these studies, addition of a 1000-fold excess of unlabelled fragment -451 to -348 bp (figure 3.31, lane 3) greatly reduced the appearance of the protein-DNA complex, whereas the fragment -347 to +50 bp (lane 4) had no effect. In addition, no competition was observed with the fragment spanning -668 to -452 bp (lane 5). These results confirmed the specificity of the observed protein-DNA interactions.

Similarly, the specificity of the -668 to -452 bp fragment was assessed. The addition of cytosol to reactions containing endlabelled -668 to -452 bp (figure 3.31 B, lane 2) caused the appearance of a band of protein-DNA (\blacktriangleleft) in addition to that of free DNA (lane 1). However, the addition of 1000-fold excess concentrations of unlabelled DNA fragments -668 to -452, -451 to -348 and -347 to +50 did not interrupt binding of DNA fragment -668 to -452 bp to receptor protein (lanes 3-5).

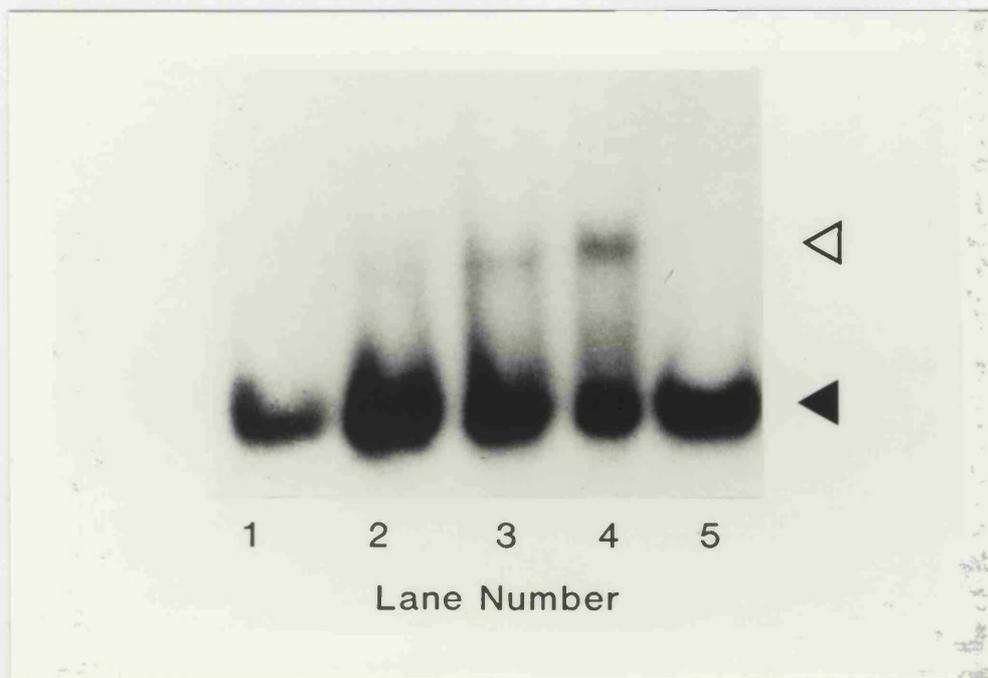


Figure 3.31 (A). Autoradiograph of a gel shift assay using labelled -451 to -348 bp fragment incubated in the absence of parathyroid cytosol (lane 1), in the presence of labelled DNA with cytosol (60 μ g) (lane 2), cytosol with a

Figure 3.30. Optimisation of gel shift assay using endlabelled -451 to -348 bp fragment incubated with increasing amounts of parathyroid cytosol: lane 1 contains no protein; lane 2-4 in the presence of 10, 20 and 40 μ g of cytosol; lane 5 contains BSA. Free DNA (▲) and protein-DNA complexes (◁) are shown.

fragments -668 to -452, lane 4 has -451 to -348 bp fragment and lane 5 has -347 to +50 bp fragment.

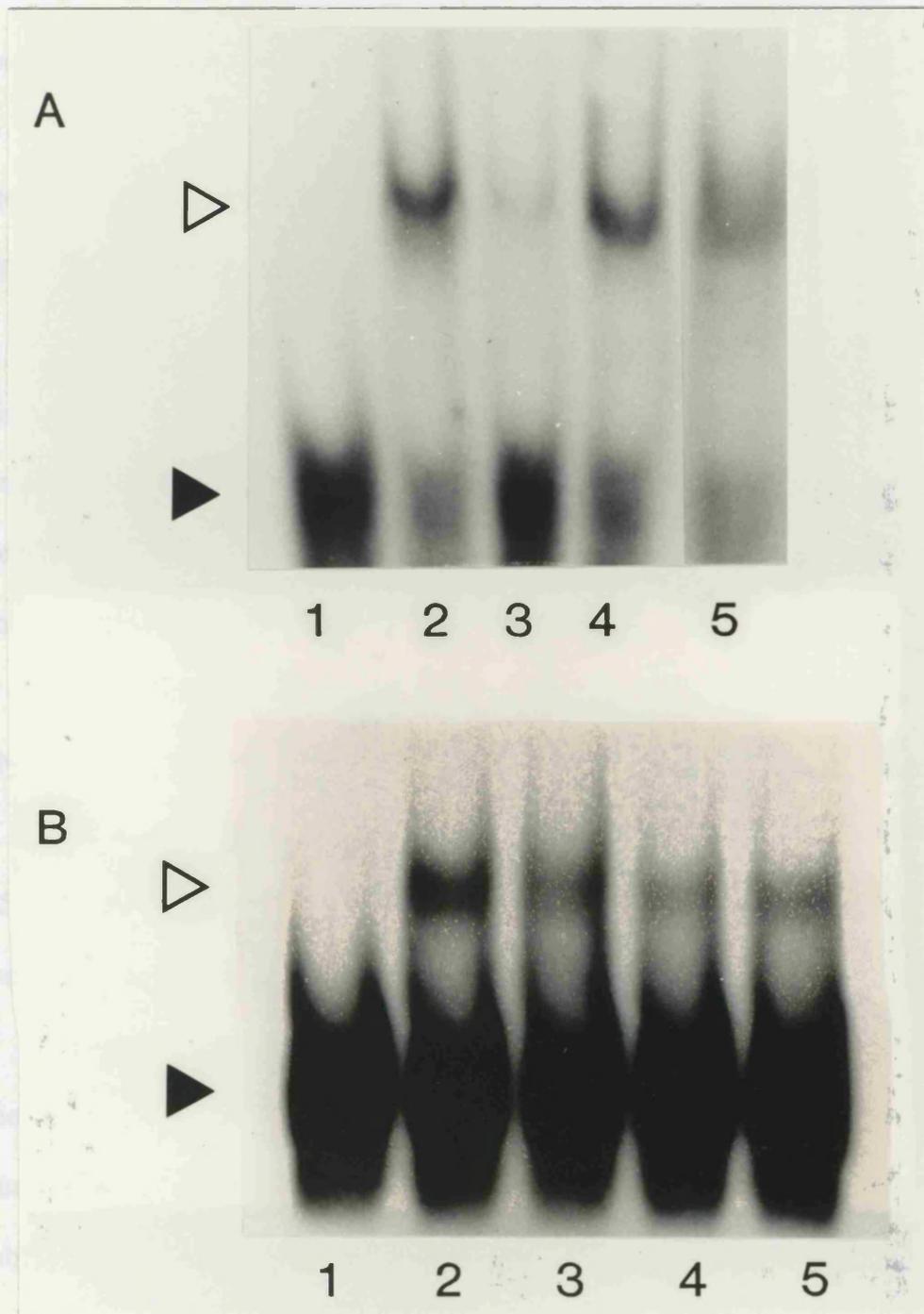


Figure 3.31 (A). Autoradiograph of a gel shift assay using labelled -451 to -348 bp fragment incubated in the absence of parathyroid cytosol (lane 1), in the presence of labelled DNA with cytosol (40 μ g) (lane 2), cytosol with a 1000-fold excess of unlabelled DNA fragments -451 to -348 bp (lane 3), -347 to +50 bp (lane 4) and -668 to -452 bp (lane 5). Free DNA (\blacktriangle) and protein-DNA complexes (\triangleleft) are shown.

(B) shows a gel shift assay using -668 to -452 bp fragment; lanes 1 and 2 are as above, lane 3 has cytosol with 1000-fold excess of unlabelled DNA fragments -668 to -452, lane 4 has -451 to -348 bp fragment and lane 5 has -347 to +50 bp fragment.

Specificity of VDR binding to the -451 to -348 bp fragment was also shown by the addition of monoclonal antibodies to VDR which abolished the protein-DNA complex, whilst non-specific rat IgG monoclonal antibodies did not affect the complex (figure 3.32).

The results of competition studies using a 1000-fold excess of unlabelled oligonucleotide consensus sequences for AP1, AP2, AP3 and SP1 in the presence of radiolabelled DNA fragments -451 to -348 bp and -668 to -452 bp are shown in figure 3.33 A and 3.33 B. The addition of cell extracts caused a band shift of the -451 to -348 bp fragment (lane 2A). However, the presence of the transcription factor consensus sequences had no effect on the protein-DNA complex formed with this fragment (lanes 3-6A). Similarly, addition of cytosol induced formation of the protein-DNA complex with the -668 to -452 bp fragment (lane 2B) but the presence of the above transcription factor consensus sequences had no effect on this complex (lanes 3-6B).

Thus, data from gel shift assays have shown specific binding of VDR to the -451 to -348 bp and -668 to -452 bp fragments, however, excess cold concentrations of -668 to -452 bp fragment failed to compete with both labelled fragments, indicating that binding of VDR to the -668 to -452 bp fragment was non-specific. Moreover, specificity of the -451 to -348 bp fragment was shown by competition with specific monoclonal antibodies.

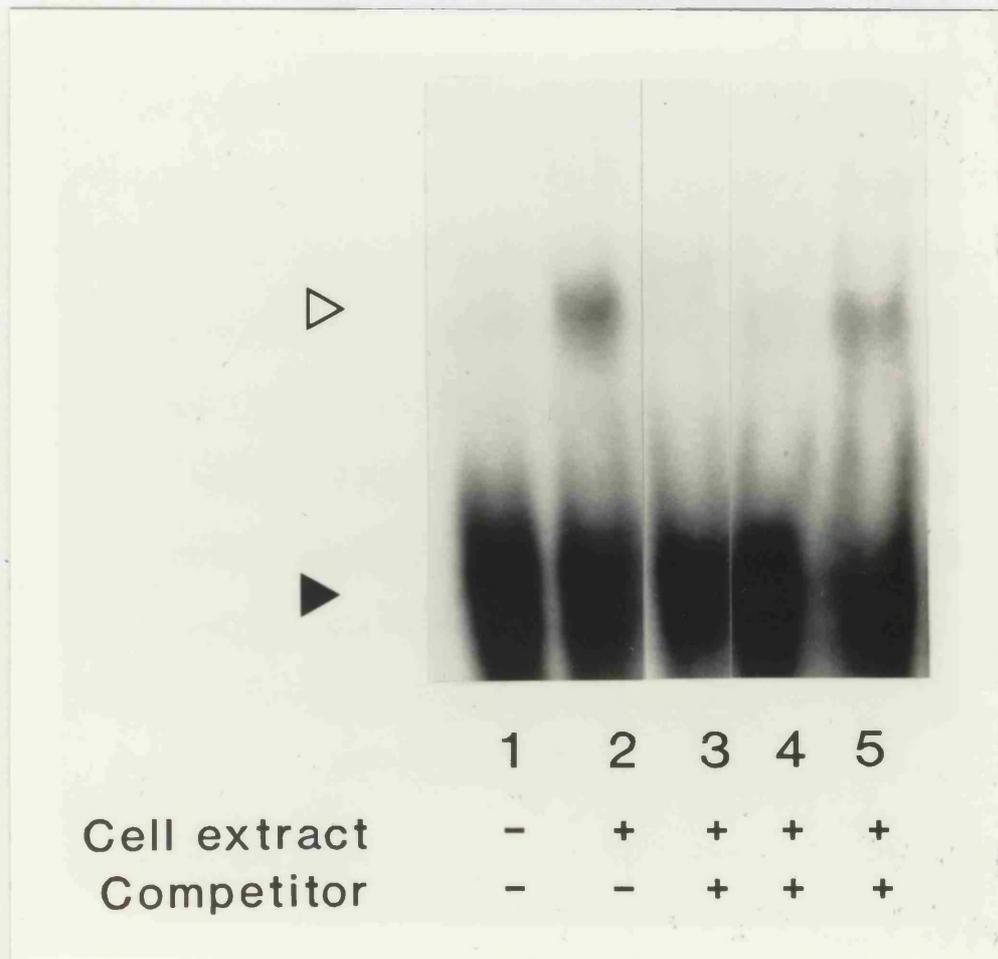


Figure 3.32. Autoradiograph of a gel shift assay using labelled -451 to -348 bp fragment incubated in the absence of parathyroid gland cytosol (lane 1), in the presence of labelled DNA and cytosol (40 μ g) (lane 2), cytosol with a 1000-fold excess of unlabelled DNA fragments -451 to -348 bp (lane 3), or 10 μ g of monoclonal antibodies 9A7 (lane 4) or 10 μ g non-specific rat IgG monoclonal antibodies (lane 5). Free DNA (\blacktriangle) and protein-DNA complexes (\triangleleft) are shown.

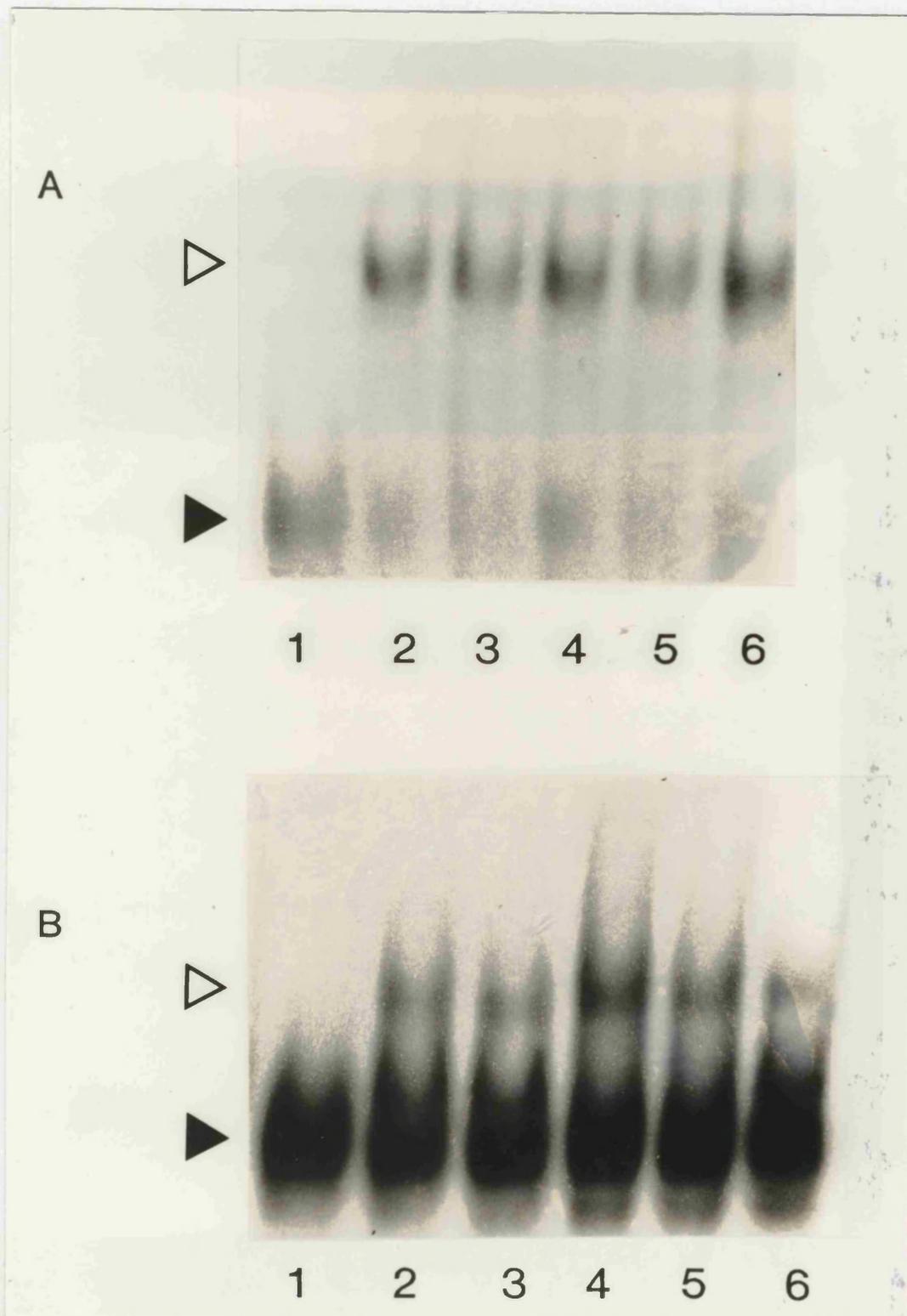


Figure 3.33 (A) shows an autoradiograph of a gel mobility shift assay using labelled -451 to -348 bp fragment in the absence of cytosol (lane 1), in the presence of labelled -451 to -348 bp fragment with cytosol (40 μ g) (lane 2) and labelled -451 to -348 bp fragment with a 1000-fold excess of unlabelled oligonucleotide sequences for transcription factors AP1, AP2, AP3 and SP1 (lanes 3-6 respectively).

(B) shows gel mobility shift assay using labelled -668 to -452 bp fragment as described for (A). Free DNA (\blacktriangle) and protein-DNA complexes (\triangleleft) are shown.

DISCUSSION

The data presented have demonstrated that the receptor for $1,25(\text{OH})_2\text{D}_3$ binds directly to the 5'-flanking region of the bovine parathyroid hormone gene. The filter binding assays indicated that there was specific binding to the -668 to +50 bp fragment which, unlike binding to the -1350 to -669 bp fragment, was not displaced by poly(dI-dC).poly(dI-dC). Digestion of the -668 to +50 bp fragment with Sau3A indicated that removal of the -100 to +50 bp sequence did not affect binding. Specificity was also shown by the absence of binding to the cDNA for the coding region of the PTH gene and likewise no binding was seen with BSA. Thus, using filter binding assays, the binding site was localised to between -668 to -100 bp upstream of the initiation site. The presence of receptors for $1,25(\text{OH})_2\text{D}_3$ in parathyroid cytosol and partially purified receptors was confirmed in Western blots using anti-receptor monoclonal antibodies. These monoclonal antibodies have been shown to shift the ^3H - $1,25(\text{OH})_2\text{D}_3$ receptor peak from 3.3S to approximately 7S in sucrose density gradients (Pike, 1984). They do not display cross-reactivity with GR or ERs, but are highly reactive with all mammalian forms of VDR (Pike *et al.* 1983).

The VDR binding site was further localised using Southwestern assays in which various fragments of the PTH promoter region were used. A single band was seen in incubations with the -451 to +50 bp, -451 to -348 bp and -668 to -452 bp fragments which was coincident with the band seen in Western blots. In contrast, no binding was seen with the -347 to +50 bp fragment. This data suggests that there are two binding domains within the

regions: -451 to -348 and -668 to -452 bp. Unexpectedly, there was no interaction with the fragment -668 to -348 bp, but it is possible that the presence of the sequence spanning -451 to -348 bp in this fragment leads to conformational changes in vitro which do not permit access of VDR to the binding domains within this fragment.

The data from the gel shift assays shown here confirmed binding of VDR to the -451 to -348 bp fragment and specificity of this reaction was shown by excess concentrations of cold -451 to -348 bp fragment which greatly reduced binding to radiolabelled fragments, whereas excess concentrations of cold -347 to +50 bp and -668 to -452 bp fragments did not compete. Specificity was also shown by the addition of monoclonal antibodies to VDR which abolished the protein-DNA complex. In contrast, non-specific antibodies failed to affect the complex.

A protein-DNA complex also formed with the -668 to -452 bp fragment in the gel shift assays although none of the fragments, namely, the -668 to -452 bp, the -451 to -348 bp or the -347 to +50 bp fragments reduced binding. The lack of competition observed with cold excess concentrations of -668 to -452 bp fragment with radiolabelled -668 to -452 bp and -451 to -348 bp fragments suggests that binding of VDR to this fragment was non-specific. This inconsistency with the results in Southwesterns, in which the -668 to -452 bp fragment bound to a protein which was coincident with VDR in immunoblots, may be due to a number of factors. For example, the cytosol added in the gel shift assays may contain an additional protein although only one DNA-binding protein was detected in Southwestern assays with cytosol.

Alternatively, the incubation conditions in the gel shift assays might have differed sufficiently from those in Southwesterns to favour binding of another protein; for instance, the incubation period in gel shift assays was shorter than in Southwestern assays.

The existence of multiple components of steroid response elements has been well documented. For instance, two glucocorticoid response elements (GRE) have been described in the tyrosine aminotransferase gene (Jantzen *et al.* 1987) in which one element has no capacity to stimulate transcription, but can enhance glucocorticoid induction in the presence of the other. In addition, the presence of at least two VDRE have been described within the rat and human osteocalcin genes (Yoon *et al.* 1988; Demay *et al.* 1990; Ozono *et al.* 1990). As with the GRE, Demay *et al.* (1990) and Ozono *et al.* (1990) have demonstrated that only the downstream VDRE is able to confer $1,25(\text{OH})_2\text{D}_3$ responsiveness whereas the upstream element is not essential but enhances the response and, in addition, the downstream element has a higher affinity for VDR.

In general, response elements such as oestrogen response elements (ERE), retinoic acid response elements (RARE) and thyroid hormone response elements (TRE) consist of two AGGTCA-like half-sites, organised as palindromes and/or direct repeats separated by gaps of varying numbers of nucleotides. Similarly, in the VDRE of the human osteocalcin gene, there are two half-site sequences, of which the upstream element (GGGTGA) resembles the half-site sequence of the ERE (Ozono *et al.* 1990). In the bovine PTH gene, two similar response elements are present placed as AGGTCA-related

direct repeats separated by six nucleotides on the lower strand with the upstream element AGGTTA within the fragment -668 to -452 bp (at -461 to -456 bp) and the downstream element AGTTCC within the fragment -451 to -348 bp (at -449 to -444 bp) as shown in figure 3.34. The presence of these elements is consistent with binding observed with the fragments as described in this study.

It has been suggested that VDREs, TREs and RAREs contain hexameric sequences that permit binding by any of the three receptors but that spacing between the half-sites determines the specificity of the HRE (Umesono *et al.* 1991; Naar *et al.* 1991). Umesono *et al.* (1991) have suggested a "3-4-5" rule in which two direct repeats of a core AGGTCA element separated by three (DR3), four (DR4) and five (DR5) nucleotide spacing was enough to discriminate between VDR, TR and RAR recognition, respectively. However, in reality, the situation is probably much more complex, since naturally occurring VDREs in particular show more variable spacing, for example, between the proximal and distal direct repeats in the human osteocalcin VDRE, a gap of seven nucleotides is present (Ozono *et al.* 1990), and four nucleotides in the rat osteocalcin VDRE (Demay *et al.* 1992a) and six nucleotides in the putative bovine PTH VDRE described above. A study presented by Freedman & Towers (1991) contradicts the "3-4-5" rule; these authors have shown that purified VDR DNA binding domains (VDR DBD) only bind with high affinity to the naturally-occurring mouse osteopontin (Spp-1) gene VDRE and not to AGGTCA core elements placed as a direct repeat with half-site spacing of three, five or as an inverted repeat with no spacing.

However, Freedman & Towers (1991) used gel mobility shift assays only and the ability of these oligonucleotide sequences to control the expression of a reporter gene was not assessed.

Towers et al. (1993) have suggested that the VDR DBD binds as a direct repeat with a spacing of three nucleotides (DR+3) cooperatively and that this effect was mediated by protein-protein contacts. The protein monomers are proposed to lie in a head-to-tail orientation on the DNA. This is in contrast to other nuclear hormone receptors such as ER and GR, which bind to palindromic half-sites as dimers in a head-to-head orientation. The amino acid residues mediating this effect lie C-terminal to zinc finger module 2 in the DNA binding domain of human VDR, termed the T-box. It has been suggested that residues in this region make contact with residues in the tip of module 1 which would orientate and stabilise the T-box of one hVDR monomer to make intermolecular contacts with module 2 of an adjacent bound monomer on the DR+3 element.

The interaction of VDR with its response elements is not fully defined, VDR may bind as a homodimer or as a heterodimer with other proteins. Studies have shown that unlike some steroid hormones, VDR dimerisation is not required for DNA interaction but heterodimerisation with a nuclear accessory factor (NAF) increases the affinity of VDR for DNA (Sone et al. 1991). NAF has been shown to increase the affinity of VDR for DNA; using VDRE affinity chromatography, Sone et al. (1991) showed that VDR forms weak interactions with the human osteocalcin VDRE in vitro binding as a monomer, whereas strong interactions which enhance binding to the response

element were due to NAF-VDR interacting as heterodimers with the VDRE.

The identity of NAF remains debatable; it behaves similarly to another class of proteins, retinoid X receptors (RXR), which have been shown to form heterodimers with VDR (Yu et al. 1991). Using transient transfection assays, Yu et al. (1991) have shown that RXR β can greatly enhance the transcriptional activity and DNA binding specificity of the TR and RAR on their respective response elements. RXRs may in turn interact with other nuclear proteins or transcription factors. Several other studies have identified proteins required by VDR for binding, for example, Nakajima et al. (1993) have identified a protein(s), receptor auxiliary factor (RAF) which is required by VDR for optimal binding to VDRE. It appears that RAF may be, or is, identical to RXRs. Their results also indicate that a C-terminal region of hVDR between cysteine-369 and arginine-402 plays an essential role in RAF and ligand binding activities.

Recently, Carlberg et al. (1993) have described two classes of response elements for vitamin D: one activated through VDR homodimers, and the other activated by VDR/RXR heterodimers. In addition, they have demonstrated that two naturally-occurring vitamin D response elements, namely that of the mouse osteopontin gene and the human osteocalcin gene, are activated differentially, that is, in RXR-dependent and RXR-independent manners, respectively. In the study presented here, the Southwestern assays demonstrated that monomeric VDR can bind directly to the bovine PTH gene in vitro. However, dimerisation with accessory proteins may be required for a hormonal response in vivo.

The possible involvement of other proteins such as transcription factors in the interaction between VDR and VDRE was also investigated in this study. Evidence exists that steroid receptors can interact cooperatively with other transcription factors to modulate target gene expression. Schule et al. (1988) have demonstrated that steroid hormone response elements can undergo synergistic interactions with a wide variety of heterologous binding sites such as those for nuclear factor 1 (NF-1), SP1 and Oct-1. The presence of an AP1 binding site within the human osteocalcin VDRE has been reported (Schule et al. 1990) and it has been suggested that it constituted an essential component of the VDRE. This site contains the consensus binding site for the Jun-Fos complex. These are products of the proto-oncogenes c-jun and c-fos which form highly stable heterodimeric protein complex (Curran & Franza, 1988). However, Ozono et al. (1990) have shown that the human osteocalcin VDRE is a complex containing both a high-affinity, proximal site recognised by VDR and a distal site that forms a consensus binding region for the AP1 protein family which is distinct from the proximal site and not essential for hormone induction. In addition, mutations within the AP1 consensus site resulted in a reduction of basal activity but had little effect on vitamin D inducibility. Thus, the proximal element is essential for functional activation by VDR and its ligand, however, both elements are required for maximal levels of promoter activity, suggesting that a functional synergism occurs between the two cis-elements. Moreover, the AP1 site in the osteocalcin promoter region is species-specific, being present in the human but not in the rat promoter region and is unlikely to be essential for hormone effect (Ozono et al. 1990).

Results of the gel shift assays indicated that there was no interaction between VDR and the consensus sequences for transcription factors AP1, AP2, AP3 and SP1 in the bovine PTH gene. In addition, the consensus sequences for AP1 and AP3 cannot be identified within the published bovine PTH gene sequence (Weaver et al. 1984).

The localisation of the VDRE to within the region spanning -485 to -348 bp of the bovine PTH gene presented in this study is consistent with the study by Okazaki et al. (1988) in which the effects of $1,25(\text{OH})_2\text{D}_3$ on human PTH gene (hPTH) transcription were shown to be mediated through the 684 bp fragment upstream of the initiation site. Demay et al. (1992b) have reported the presence of a VDRE in the region -125 and -101 bp in the human PTH gene which competes with the rat osteocalcin VDRE for receptor binding in gel mobility shift assays and confers responsiveness to $1,25(\text{OH})_2\text{D}_3$. However, this data does not exclude the possibility of other VDRE in the PTH gene, since the VDRE in the PTH gene is not necessarily identical to that in the osteocalcin gene as $1,25(\text{OH})_2\text{D}_3$ suppresses transcription in the former but stimulates gene activity in the osteocalcin gene.

Thus, the results from these studies have revealed two VDR binding domains within the bovine PTH gene in the regions spanning -668 to -452 bp and -451 to -348 bp. Combining these data with previous results from filter binding assays in which the binding domain was localised within the -485 to -100 bp, the binding sites can now be further defined to within -485 to -452 and -451 to -348 bp (Hawa et al. in press). Sequence-specific characterisation using gel mobility shift assays have demonstrated that these fragments bind to

VDR with differing affinities. Functional characterisation of these fragments in transient expression assays can provide a further insight into promoter activity and regulation by $1,25(\text{OH})_2\text{D}_3$ and these will be discussed in the following chapter.

CHAPTER 4

FUNCTIONAL CHARACTERISATION OF FRAGMENTS OF THE BOVINE PTH GENE

INTRODUCTION

The study of the physical interactions of VDR with the bovine PTH gene, as described in chapter 3 using *in vitro* DNA binding assays (Southwestern and gel mobility shift assays), has demonstrated that VDR interact directly with the 5'-flanking region of the gene. The binding sites were localised to within two fragments spanning the region -485 to -452 and -451 to -348 bp upstream of the initiation site. However, Southwestern and gel shift assays may not reflect complex interactions which occur in cells and thus these DNA fragments were characterised further in functional assays. PTH fragments were linked to a reporter gene, transfected into opossum kidney cells and the ability of these fragments to mediate $1,25(\text{OH})_2\text{D}_3$ -responsiveness was determined.

It is possible to study the effects of regulatory DNA sequences within the promoter and 5'-flanking regions for transcriptional activity *in vivo* by linking sequences upstream of the coding region for a reporter gene and introducing the constructs into eukaryotic cells in culture. DNA can be transferred into eukaryotic cells by transient transfection in which plasmid DNA does not become integrated into the host cell chromatin. As the cells are incubated, the transfected DNA is progressively lost from them due to degradation since DNA which is not integrated into nuclear material is not replicated along with the host cell chromosomes. Using this technique, expression of incoming DNA can be monitored as early as 12 hours after uptake and as late as 72 hours after transfection (Gorman, 1985). This approach allows rapid screening for the production of gene products coded by the plasmid and is

usually used to perform in vivo analysis of regulatory sequences in genes that control transcription and consequently was used in this study. Alternatively, stable cell lines can be produced, in which DNA is incorporated into the host chromatin permanently, however, since a number of PTH fragments were being characterised in the study described here, transient transfections were preferred.

Several methods of transferring cloned DNA into cultured eukaryotic cells have been developed, including diethylaminoethyl dextran (DEAE-dextran) (McCutchan & Pagano, 1968), calcium phosphate (Wigler et al. 1977), electroporation (Potter, 1984) and liposomes (Felgner et al. 1987). The earliest of these methods of transiently introducing DNA into cells was to mix DNA and DEAE-dextran, an inert carbohydrate polymer (dextran) linked to a positively charged chemical group (DEAE). DNA binds to DEAE-dextran via its phosphate groups and interacts with plasma membranes thus bringing DNA close to the cell surface ready for uptake. Although this method is relatively simple, it is inefficient for many types of cells and other techniques have been developed. The calcium phosphate technique is the most widely used method to introduce DNA into cultured eukaryotic cells. Exogenous DNA is mixed with calcium chloride and is added to a solution containing phosphate ions, forming a calcium phosphate-DNA co-precipitate which is readily taken up by cells. The advantage of this technique is that DNA is efficiently transferred into cells, however, the contact time between the cells and calcium phosphate required is approximately 16-24 hours. Plasmid DNA can also be introduced into cultured cells by electroporation, in which cells are

subjected to a brief electrical pulse that causes holes to open transiently in the membranes through which DNA enters directly into the cytoplasm. The efficiency of transfection by electroporation is influenced by a number of factors, including the strength and time of the applied electric field, and the temperature at which electroporation is performed. However, a major disadvantage of this technique is that a large number of cells are required since the rate of cell death is high. Thus the method of choice in this study was the incorporation of DNA into artificial lipid vesicles, or liposomes. A commercially available liposome, lipofectin, which is a 1:1 (w/w) formulation of the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) was used. These form positively charged liposomes which interact spontaneously with DNA or RNA to form complexes, adsorb to cell surfaces and fuse with the plasma membrane, thus efficiently transferring their contents directly into the cytoplasm. Unlike electroporation, this method does not require a large number of cells or large amounts of DNA and is a simple and a fast method of transferring DNA into cultured cells.

A variety of bacterial genes are available for functional studies and have been used in eukaryotic expression vectors. These include the gene encoding chloramphenicol acetyltransferase (CAT) (Gorman *et al.* 1982), luciferase (Gould & Subramani, 1988), and the E.coli lacZ gene that encodes for β -galactosidase (Sambrook *et al.* 1989), all of which exhibit a specific enzymatic activity distinguishable from mammalian activity. A popular and recently developed reporter gene is the firefly luciferase gene (Gould and Subramani,

1988) which catalyses the reaction:



The light which is emitted is directly proportional to expression of the reporter gene and is measured using a luminometer. This method is used particularly for analysing the transcriptional activity of weak promoters and usually in cells that do not readily accept or express exogenous DNA. In addition, this assay permits the use of small quantities of plasmid DNA and does not require radiochemicals.

Human growth hormone (hGH) has also been used as a reporter gene in mammalian cells (Selden *et al.* 1986). This differs qualitatively from the other commonly used reporter proteins in that it is secreted from cells. Thus it can be measured using a sample of culture medium without the need to prepare cell extract. However, it can only be used in secretory cells in which it offers several advantages over the more conventional intracellular reporter genes in that, after the determination of GH in the medium, the cells can be used for RNA isolation. However, the GH assay should be used with caution, since it is based on the assumption that the levels of secreted and intracellular protein are parallel.

The reporter gene of choice in this study was CAT since it is easily measured in cell extracts and did not require additional equipment. CAT is a bacterial gene that inactivates chloramphenicol by transferring an acetyl group to one or both hydroxyl groups present on chloramphenicol resulting in mono- and diacetylated derivatives. The acetylated and unacetylated products

possess different polarities in organic solvents, enabling their separation by silica thin-layer chromatography (TLC). When putative regulatory sequences in a CAT construct are transfected into cultured eukaryotic cells, transcripts initiate at the eukaryotic promoter and proceed through the CAT gene. The resulting transcripts are translated into CAT enzyme protein by the translational machinery of the host cell and the changes in the level of enzymatic activity is a measure of the ability of the promoter to regulate transcription. The CAT gene is not found in eukaryotic cells so there is little or no background activity. This is an indirect but widely used assay of the activity of promoters that control transcription and has been used for studying regulatory sequences of a number of genes, including osteocalcin, human and avian PTH (McDonnell *et al.* 1989; Demay *et al.* 1992a; Russell *et al.* 1992).

A number of commercial CAT plasmids are available which contain the CAT coding region, simian virus (SV40) promoter and/or enhancer sequences built into a pUC19 or pBR322 plasmid backbone, the gene for antibiotic resistance (usually ampicillin or tetracyclin) and the prokaryotic origin of replication. In addition, these vectors generally contain a unique multiple cloning site upstream of the reporter gene for insertion of test DNA fragments. Most cell types can be used for transfection of CAT plasmids, however, COS cells which contain the large T antigen of the SV40 virus produce high background levels of CAT enzyme and therefore cannot be used with CAT plasmids containing the SV40 origin of replication. In this study, cells derived from the proximal tubule epithelium of the opossum kidney were used which have been used in transient transfection studies with fragments of

the avian PTH gene in CAT constructs (Russell *et al.* 1992).

AIM OF THIS STUDY

The aim of this study was to investigate whether the DNA fragments containing the putative VDRE described in chapter 3 were transcriptionally active and responsive to $1,25(\text{OH})_2\text{D}_3$. To determine this, they were placed upstream of the CAT gene and transfected into OK cells using lipofectin in transient gene expression assays. Since OK cells express a low number of VDR (Russell *et al.* 1992), VDR cDNA in the expression vector, pSVK3 was co-transfected. This vector has been used in expression studies in which the VDR/pSVK3 plasmid was cotransfected into VDR-deficient CV-1 cells, resulting in induction of CAT activity in the presence of $1,25(\text{OH})_2\text{D}_3$ (Kristjansson *et al.* 1993).

In this chapter, methods for the generation of CAT constructs will be described separately from those that were used specifically for optimisation of transfection which will be discussed with the results obtained.

METHODS

Synthesis of recombinant CAT constructs

Competent *E. coli* HB101 cells were transformed with pCAT enhancer plasmid (4610 bp) purchased from Promega Ltd (Madison, WI, USA), isolated and purified by large-scale preparation as described in Appendix I.

pCAT enhancer plasmid (10 μ g) was digested with PstI (5 Units/ μ g) in buffer H containing 5 mM spermidine at 37°C for 16 hours. After digestion, the plasmid was purified by phenol:chloroform extraction before dephosphorylation using calf intestinal alkaline phosphatase (CIP) as described in Appendix I.

Generation of DNA fragments

The DNA fragments generated by SspI digestion of the -668 to +50 bp region, that is, the -347 to +50, -668 to -452 and the -451 to -348 bp fragments as described in chapter 3, and the -668 to +50 bp fragment were blunt-ended (see removal of 3' protruding ends) and ligated into linearised pCAT enhancer plasmid as described in Appendix I. These fragments in the CAT plasmid will be referred to as pCAT.PTH400, pCAT.PTH250, pCAT.PTH100, and pCAT.PTH700, respectively and are shown in figure 4.1. The numbers 400, 250, 100 and 700 refer to the approximate length of the DNA fragment. Since the -668 to -452 and the -451 to -348 bp fragments lack the TATA region, a fragment of the bovine PTH gene from -100 to -2 bp containing this region was directionally cloned firstly into pBluescript

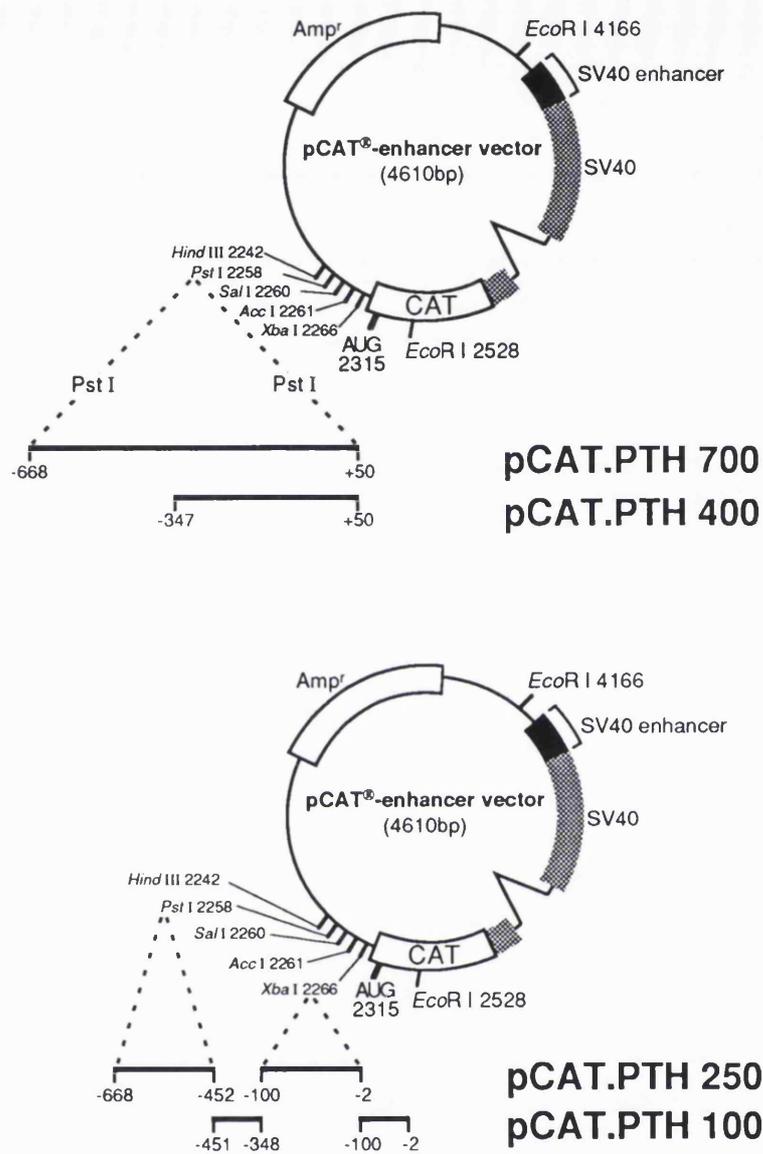


Figure 4.1. The PTH gene fragments were inserted into the PstI site of pCAT enhancer plasmid and are designated as follows: **pCAT.PTH700**: -668 to +50 bp; **pCAT.PTH400**: -347 to +50 bp; **pCAT.PTH250**: -668 to -452 with -100 to -2 bp; **pCAT.PTH100**: -451 to -348 with -100 to -2 bp.

plasmid (this was done so that a number of restriction sites were retained for use as diagnostic tests), followed by ligation into the XbaI site of pCAT enhancer plasmid as shown in figure 4.1.

The PTH fragments can insert into the pCAT plasmid in either orientation with respect to the CAT gene and diagnostic digestions were performed using a combination of restriction enzymes in pCAT plasmid and insert DNA fragments. Usually 6-10 clones were digested and the sizes of fragments were calculated from the published bovine PTH gene sequence (Weaver *et al.* 1984), whilst those observed were determined from DNA molecular size markers.

i) pCAT.PTH700

Six clones containing the -668 to +50 bp region in pCAT plasmid were digested with PstI (figure 4.2 A, lanes 2-7) which removes the insert fragment, and with XbaI (figure 4.2 A, lanes 8-13) and HindIII (figure 4.2 B, lanes 1-6) which both indicate the orientation of the insert DNA with respect to the CAT gene. Lane 1 represents linearised pCAT plasmid.

Digestion with PstI gave two fragments of 4900 and 800 bp as seen in lanes 2, 3, 6, and 7 (figure 4.2 A). A useful clone in the sense orientation would be one which when digested with XbaI gave a fragment of 5400 bp and in the antisense orientation would be one that yielded fragments of 4600 and 800 bp. This was observed in lanes 9, 12 and 13, and lane 8 (A), respectively. Digestion with HindIII is also a useful test for orientation and should give fragments of 4740 and 660 bp which were observed in lanes 2,

5 and 6 (B). Thus, the change in level 2 (small number) and level 5 (medium

number) were similar and similar to the change in level 3 (large number) and level 4 (medium

number)

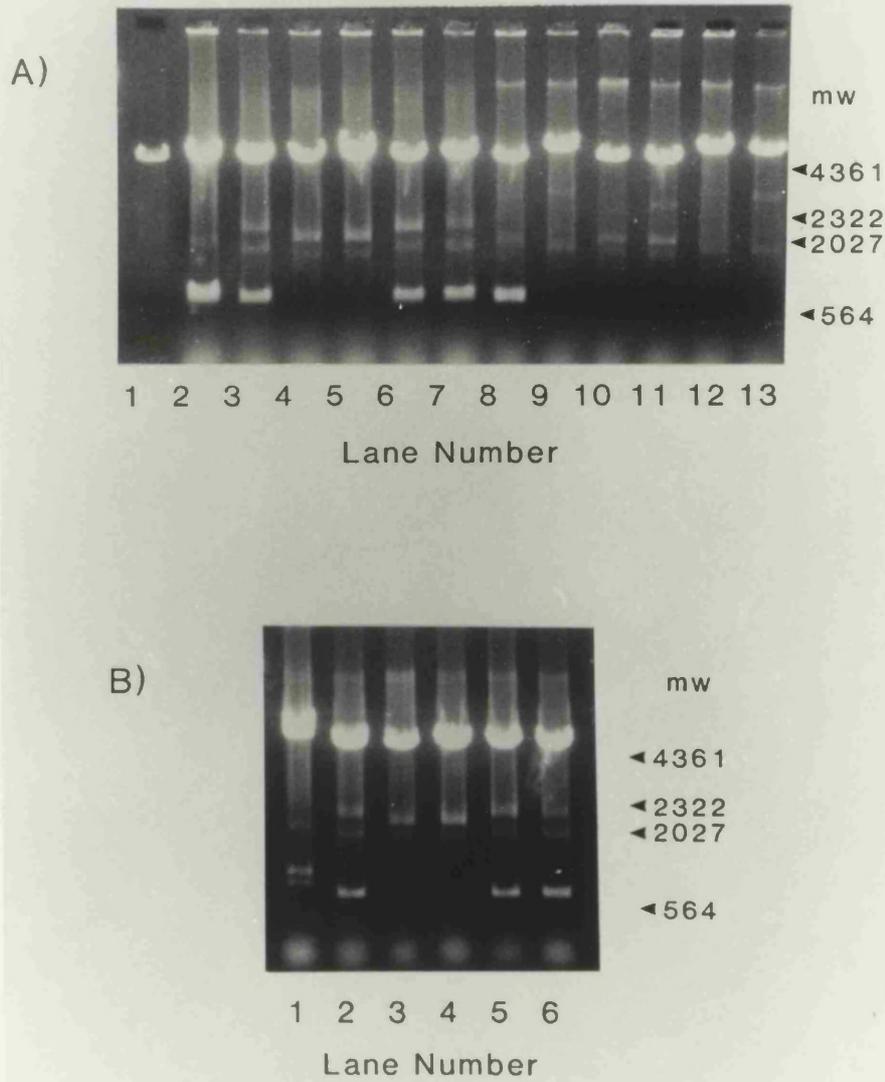


Figure 4.2. Digestion of -668 to +50 bp region in pCAT plasmid with PstI (A lanes 2-7), XbaI (A lanes 8-13) and HindIII (B lanes 1-6). Lane 1 represents linearised pCAT enhancer plasmid. Molecular size markers are shown on the right.

5 and 6 (B). Thus, the clones in lane 2 (sense construct) and lane 8 (antisense construct) were grown and purified by caesium chloride ultracentrifugation and subsequently used in transfection studies.

ii) pCAT.PTH400

Eight clones containing the -347 to +50 bp fragment were digested with HindIII (figure 4.3) which is present in the PTH gene insert and thus can be used to indicate the orientation of the insert. A useful clone in the sense orientation would be one which gave a fragment of approximately 320 bp in addition to one at 4700 bp. The only clone which met both these criteria was seen in lane 2, indicating that this fragment had inserted in the sense orientation with respect to the CAT gene and was subsequently used in transfection studies.

iii) pCAT.PTH250

Eight clones containing the -668 to -452 bp fragment in pCAT plasmid were digested with XbaI (figure 4.4 A) and EcoRI + HindIII (B). Lane 1 in (A) represents pCAT plasmid digested with EcoRI + HindIII. Digestion with XbaI which linearises the construct gave a major band at 4900 bp observed in lanes 3, 5, 7, 8 and 9 (figure 4.4 A). A test which would provide an indication of whether insert DNA was present, is the double digestion using EcoRI + HindIII which should give fragments of 2800, 1600 and 500 bp as seen in lanes 3-9 (figure 4.4 B). As a control, pCAT plasmid digested with EcoRI + HindIII gave fragments of 2800, 1600 and 300 bp (lane 1 B),

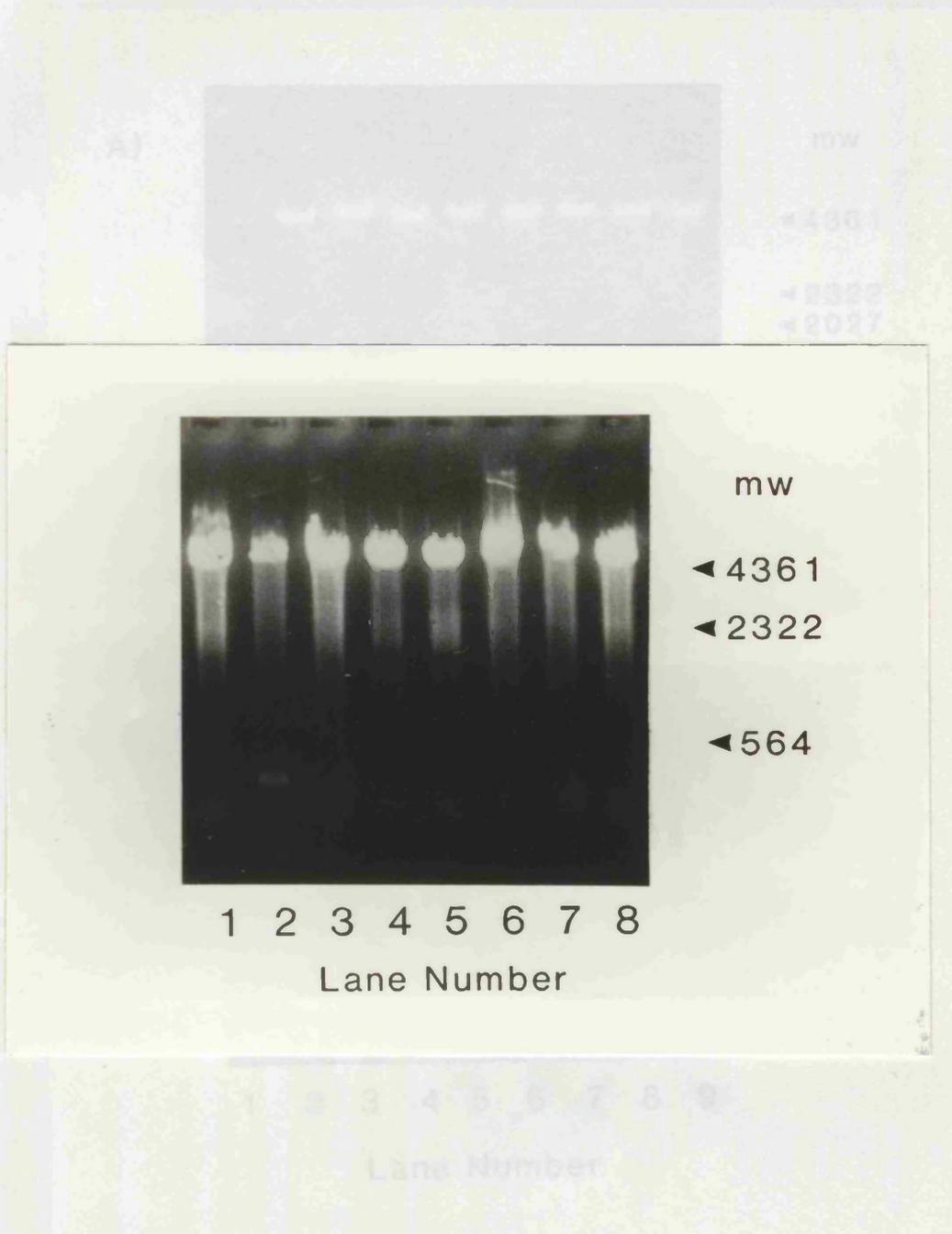
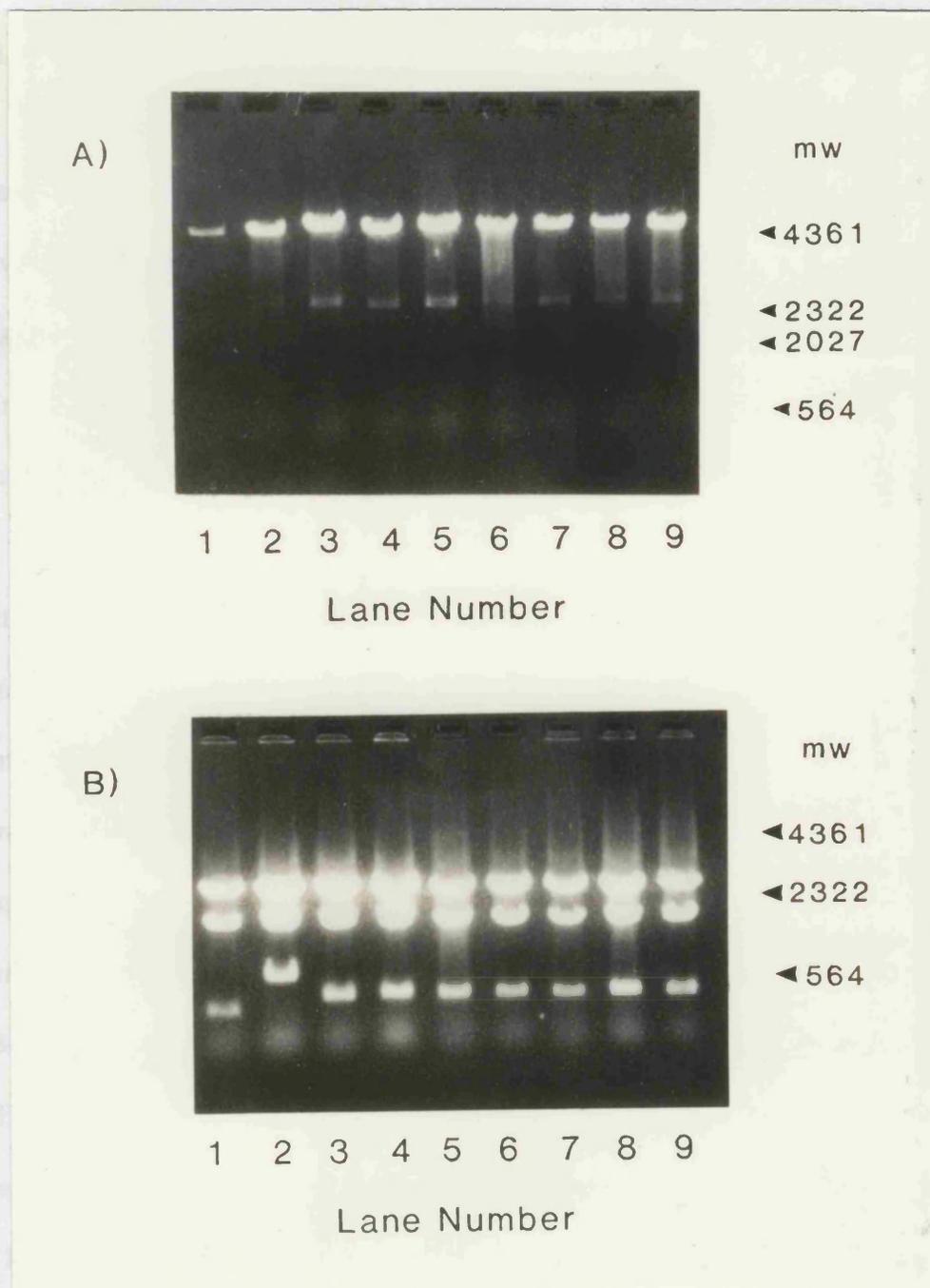


Figure 4.3. Restriction enzyme digestion of the -347 to +50 bp region in pCAT plasmid with HindIII. Molecular size markers are indicated on the right.

indicating the absence of an early DNA...



ng each) were sequenced using T7 DNA polymerase as described in chapter

3 and electrophoresed on a 4% polyacrylamide gel. Figures 4.6 and 4.7 show

Figure 4.4. Restriction enzyme digests of eight clones of -668 to -452 in pCAT plasmid with XbaI (A), EcoRI + HindIII (B). Lane 1 in (B) represents pCAT enhancer plasmid digested with EcoRI + HindIII. Molecular size markers are indicated on the right.

indicating the absence of an insert DNA.

iv) pCAT.PTH100

Similarly, nine clones containing the -451 to -348 bp fragment in pCAT plasmid were digested with EcoRI + HindIII (figure 4.5, lanes 2-10) which gave three fragments including a 400 bp fragment as seen in lanes 2 and 8 (figure 4.5). Lane 1 represents pCAT plasmid digested with EcoRI + HindIII. In the absence of insert DNA, a fragment of 300 bp was seen, for example, digestion of pCAT plasmid with EcoRI + HindIII (lane 1).

However, digestion of the pCAT.PTH250 and pCAT.PTH100 constructs with restriction enzymes does not give an indication of the orientation of the -668 to -452 and -451 to -348 bp fragments with respect to the CAT gene. Thus these constructs were digested with EcoRI + HindIII and cloned directionally into the EcoRI + HindIII site of pBluescript SK (+) for sequencing as described in chapter 3. These sites were chosen so that a number of restriction sites were retained with the fragment after ligation into pBluescript and thus available for test digestions. After transformation of HB101 cells, the clones were grown in 50 ml LB-broth, the unlinearised plasmid was separated on a 0.8% agarose gel and purified using a Gene Clean II kit as described in Appendix I. Two clones of each of these constructs (2 μ g each) were sequenced using T7 DNA polymerase as described in chapter 3 and electrophoresed on a 6% polyacrylamide gel. Figures 4.6 and 4.7 show examples of autoradiographs following sequencing of each of these clones. Having confirmed the orientation of the clones, they were grown in 500 ml



Figure 4.5. Digestion of nine clones containing the -451 to -348 bp fragment in pCAT plasmid with EcoRI + HindIII (lanes 2-10). Lane 1 represents pCAT plasmid digested with EcoRI + HindIII. Molecular size markers are indicated on the right.



Figure 4.6. Autoradiograph following sequencing of the -668 to -452 bp fragment in pCAT plasmid inserted into pBluescript plasmid. A small region of the PTH insert is indicated.

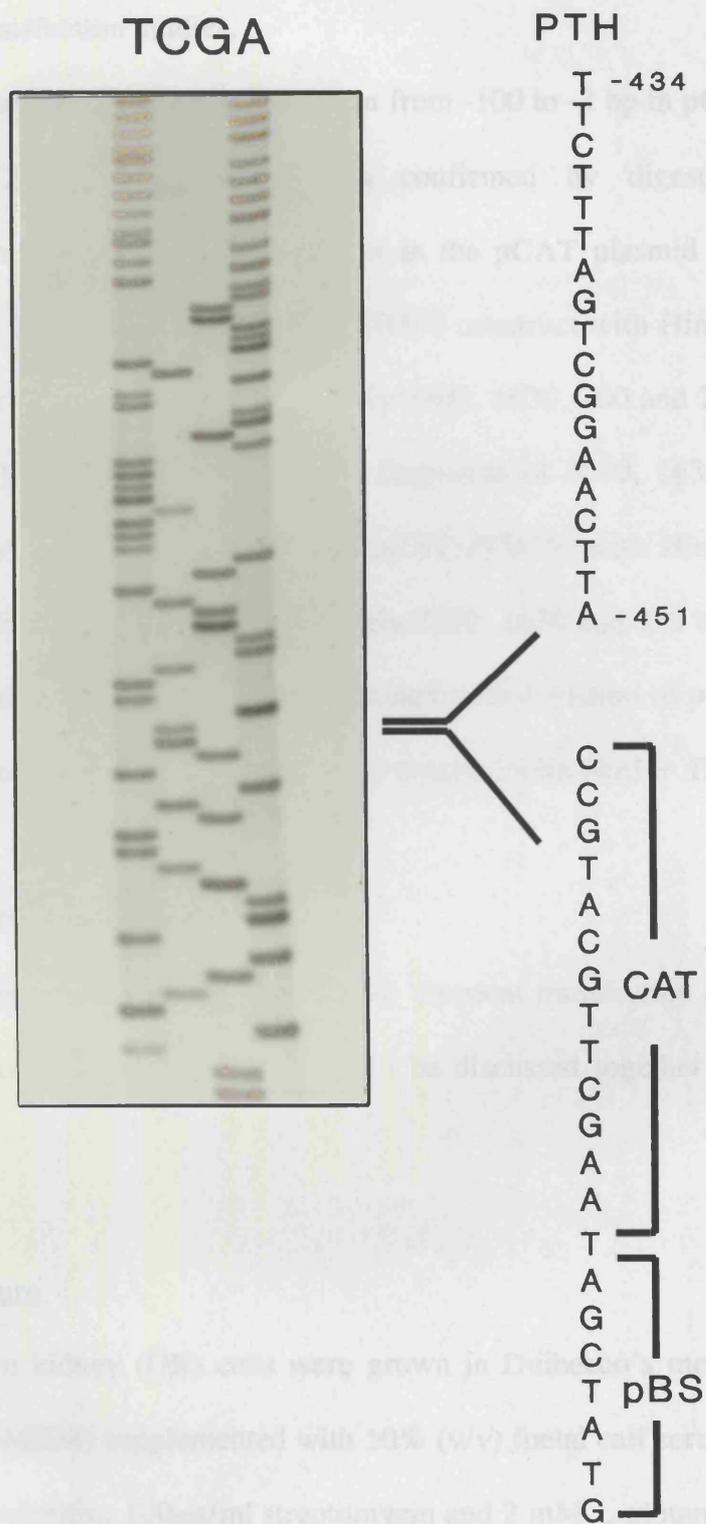


Figure 4.7. Autoradiograph following sequencing of the -451 to -348 bp fragment in pCAT plasmid inserted into pBluescript plasmid. A small region of the PTH fragment is shown.

LB-broth and purified by caesium chloride ultracentrifugation before use in transient transfection studies.

The presence of the promoter region from -100 to -2 bp in pCAT.PTH100 and pCAT.PTH250 constructs was confirmed by digestion using a combination of restriction sites present in the pCAT plasmid and the PTH fragments. Digestion of the pCAT.PTH100 construct with HindIII + EcoRI yielded four fragments of approximately 2800, 1630, 300 and 200 bp (figure 4.8, lane 1) and with NotI + EcoRI fragments of 2950, 1630 and 400 bp were observed (lane 2). Digestion of pCAT.PTH250 with HindIII + EcoRI yielded three fragments of approximately 2800, 1630 and 330 bp (lane 3). A similar pattern of fragments to that obtained with digestion of pCAT.PTH100 was observed when pCAT.PTH250 was digested with NotI + EcoRI (lane 4).

Transient transfection assays

The optimisation of methods used for transient transfection assays and the results obtained from those studies will be discussed together in the results section.

Tissue culture

Opossum kidney (OK) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 100 Units/ml penicillin, 100µg/ml streptomycin and 2 mM L-glutamine (complete DMEM) at 37°C and 5% CO₂.

Promotion of cell growth for CAT and β -galactosidase activities

Cells were washed three times with phosphate-buffered saline (PBS) and incubated with 1 ml 40 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA and 15 mM NaCl (TEN) for 5 minutes at 20°C. Cells were scraped,



and an ELISA followed by autoradiography was performed.

The assay used in this study is a modification of the method using acetyl

CoA as a substrate for CAT (Seed &

Figure 4.8. Digestion of pCAT.PTH100 and pCAT.PTH250 constructs containing the PTH promoter region from -100 to -2 bp with HindIII + EcoRI (lanes 1 and 3, pCAT.PTH100) and NotI + EcoRI (lanes 2 and 4, pCAT.PTH250). Molecular size markers are shown on the right.

Preparation of cell extract for CAT and β -galactosidase activities

Cells were washed three times with phosphate-buffered saline (PBS) and incubated with 1 ml 40 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA and 15 mM NaCl (TEN) for 5 minutes at 20°C. Cells were scraped, transferred to microfuge tubes and centrifuged at 10 000 g for 10 minutes at 4°C. The resulting pellet was resuspended in 150 μ l of 0.25 M Tris-HCl (pH 8.0). The cell extract was subjected to 3 freeze-thaw cycles, with vortexing after each thaw cycle. After centrifugation, 25 μ l cell extract was removed for measuring β -galactosidase activity and protein estimation. The remaining extract was heated at 60°C for 10 minutes to inactivate endogenous acetylases. The extract was centrifuged at 10 000g at 4°C for 10 minutes, the supernatant transferred to fresh tubes and stored at -70°C.

(i) CAT assay

CAT activity can be measured by two alternative methods, one is based on liquid scintillation counting (LSC) and the other by silica gel thin layer chromatography (TLC). In both methods, cell extracts are incubated in a reaction mix containing ^3H or ^{14}C -labelled chloramphenicol and n-acetyl CoA or n-butyryl CoA, however, the LSC method was found to be less sensitive and so TLC followed by autoradiography was preferred.

The assay used in this study is a modification of the method using acetyl CoA in which butyryl CoA is substituted as a substrate for CAT (Seed & Sheen, 1988). Cell extracts were diluted in 0.25 M Tris-HCl (pH 8.0) and the reaction was performed as follows:

cell extract	40 μ l
0.25M Tris-HCl (pH8.0)	70 μ l
¹⁴ C-chloramphenicol (0.025mCi)	10 μ l
n-butyryl CoA (5 mg/ml)	5 μ l
Total volume	125 μ l

The reaction was incubated at 37°C for 16 hours, samples were extracted with ethyl acetate (500 μ l) and vortexed for 1 minute, followed by centrifugation at 10 000 g for 3 minutes. The upper organic phase was transferred to a fresh tube and evaporated to dryness under vacuum. The residue was resuspended in 10 μ l of ethyl acetate and dotted onto silica gel-coated TLC plates. The plates were dried before placing in a tank pre-equilibrated with chloroform:methanol (97:3) and the solvent front was allowed to move approximately 75% of the distance in a closed tank. The plates were removed, air-dried and exposed to X-ray film for 24 hours at 20°C. The resulting autoradiographs were scanned on a densitometer at 525 nm and CAT activity was related to protein concentration. Throughout this study, protein concentrations of cell extracts were determined using the method of Lowry *et al.* (1951).

Figure 4.9 (A) is an example of an autoradiograph of a CAT standard curve separated by TLC showing the unacetylated chloramphenicol (S) at the line of origin and the two faster migrating dots, mono-acetylated (M) and diacetylated (D) forms of chloramphenicol. Following densitometry, a linear relationship between the amount of CAT activity (Units/ μ l) and densitometer readings was observed as shown in (B).

In vitro studies

Cell extracts diluted in distilled water (1:50 v/v) were mixed with 150 μ l of

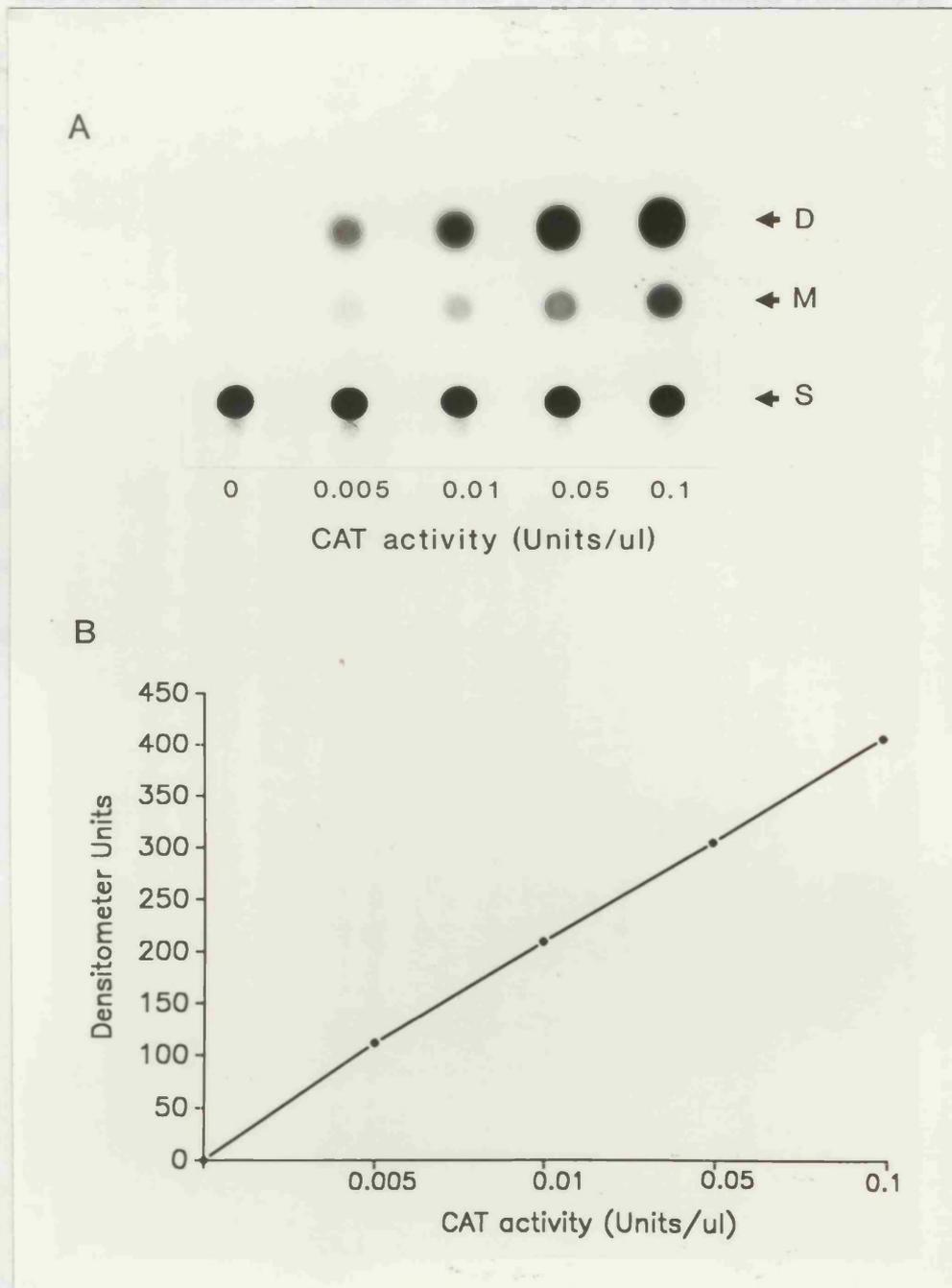


Figure 4.9. (A) Autoradiograph of a CAT standard curve separated by TLC showing the substrate chloramphenicol (S) mono- (M) and diacetylated (D) forms of chloramphenicol. (B) shows the linear relationship between CAT activity and densitometer readings of total products.

(ii) β -galactosidase assay

Cell extracts diluted in distilled water (150 μ l) were mixed with 150 μ l of 2X assay buffer (120 mM Na_2HPO_4 , 80 mM NaH_2PO_4 , 2 mM MgCl_2 , 100 mM β -mercaptoethanol and 1.33 mg/ml o-nitrophenyl- β -D-galactopyranoside) in duplicate. The reaction was incubated at 37°C for 30 minutes and stopped by the addition of 1 M sodium carbonate (500 μ l). The absorbance of the reaction was measured at 420 nm. A standard curve was prepared by serial dilutions of 1 U/ μ l of β -galactosidase to 1.25×10^{-6} U/ μ l (figure 4.10). The β -galactosidase concentration in cell extract samples was determined by comparison to the standard curve. The β -galactosidase activity in the cell extracts was within the range 5.0 - 12.0×10^{-4} Units/ μ l.

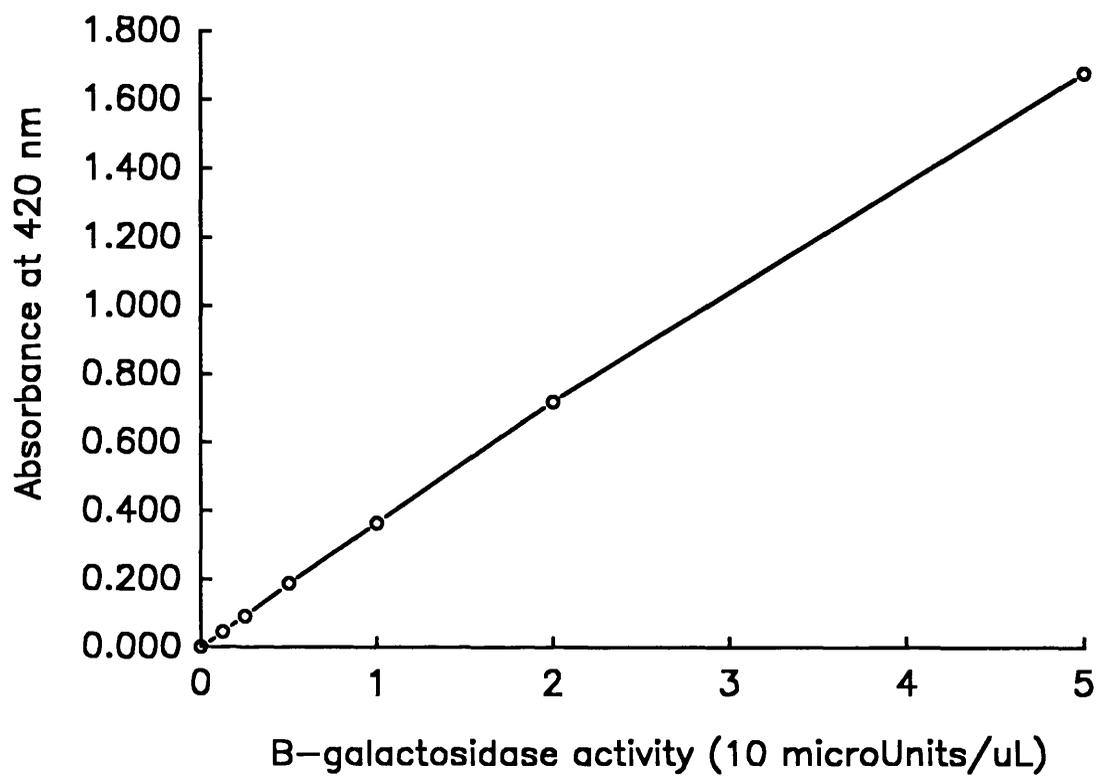


Figure 4.10 Standard curve for determination of β -galactosidase activity.

RESULTS

1. Optimisation of transfection

Growth curve for opossum kidney cells

A growth curve for OK cells was set up in order to determine the optimal cell number needed for transfections. When cells are seeded into petri dishes (2.5×10^4 cells per 60 mm petri dish), they enter a lag phase during which time they adjust to their new environment, followed by a period of exponential growth as seen by day 3 (figure 4.11), whereby the cells begin to multiply rapidly, that is, the logarithmic phase. Finally, the cells enter a period of reduced or no growth after they become confluent (stationary phase) as seen at day 7. The growth rate eventually slows down as the cells begin to die due to toxic build up and lack of nutrients.

The optimal cell number was kept constant in all experiments in order to obtain reproducible results and in subsequent experiments, cells were plated 24 hours before transfection at a density of 2.5×10^5 per 60 mm dish, that is, in the log phase of growth as seen within 2-3 days (figure 4.11). Cells were typically 50-60% confluent prior to transfections. The number of times cells were subcultured was noted since their responsiveness to $1,25(\text{OH})_2\text{D}_3$ changes.

Optimisation of amount of lipofectin

To introduce DNA into OK cells, lipofectin (Gibco BRL, UK) was used. The amount of lipofectin reagent required for transfection was optimised

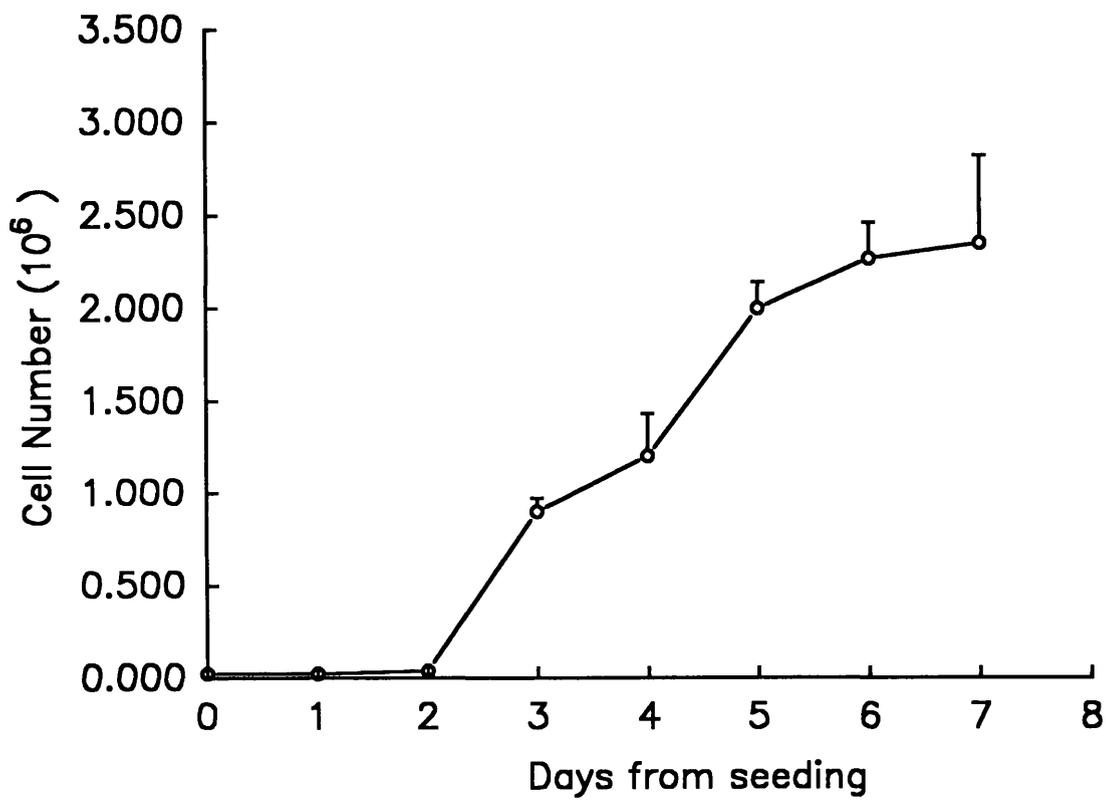


Figure 4.11 Seven day growth curve for adherent opossum kidney cells. Cells were seeded at 2.5×10^4 per 60 mm petri dish and the number of viable cells were determined by trypan blue exclusion.

keeping the concentration of DNA and incubation time constant. The pCAT.PTH700 construct (5 μg) in the sense orientation and pSV β -galactosidase plasmid (5 μg , see Appendix III for plasmid map) were co-transfected into adherent monolayers of OK cells. Lipofectin reagent (1 mg/ml; 5, 10, 20 and 50 μl) was mixed in an equal volume of serum-free DMEM containing DNA (10 μg) and DNA-lipofectin complexes were allowed to form at 20° C for 15 minutes. Cells were washed twice with serum-free DMEM prior to addition of DNA-lipofectin complexes. Plates were set up in quadruplicate, two for transfection with lipofection and two for cell number which was determined by trypan blue exclusion. In addition, two plates were treated as controls, that is, untransfected. After incubation for 6 hours at 37°C and 5% CO₂, medium was replaced with complete DMEM and the cells incubated for 48 hours at 37°C and 5% CO₂. Cells were harvested 48 hours post-transfection and cell extracts were prepared for CAT and β -galactosidase activities. CAT and β -galactosidase activity in the cell extracts were measured in duplicate as described earlier and CAT activity was quantified by densitometry.

Lipofectin (50 μl) resulted in a strong signal with overnight exposure, however, this caused greater cell death, that is, 0.59 X 10⁶ cells/ml compared to 2.1 X 10⁶ cells/ml in cells not exposed to lipofectin, indicating that this concentration was toxic to the cells (figure 4.12). Transfection with lipofectin (20 μl) gave an approximately 4-fold greater CAT activity than 10 μl , however, cell death was higher (1.69 versus 2.5 X 10⁶ cells/ml respectively). Although lipofectin (5 μl) apparently gave a 1.6-fold increase in CAT activity

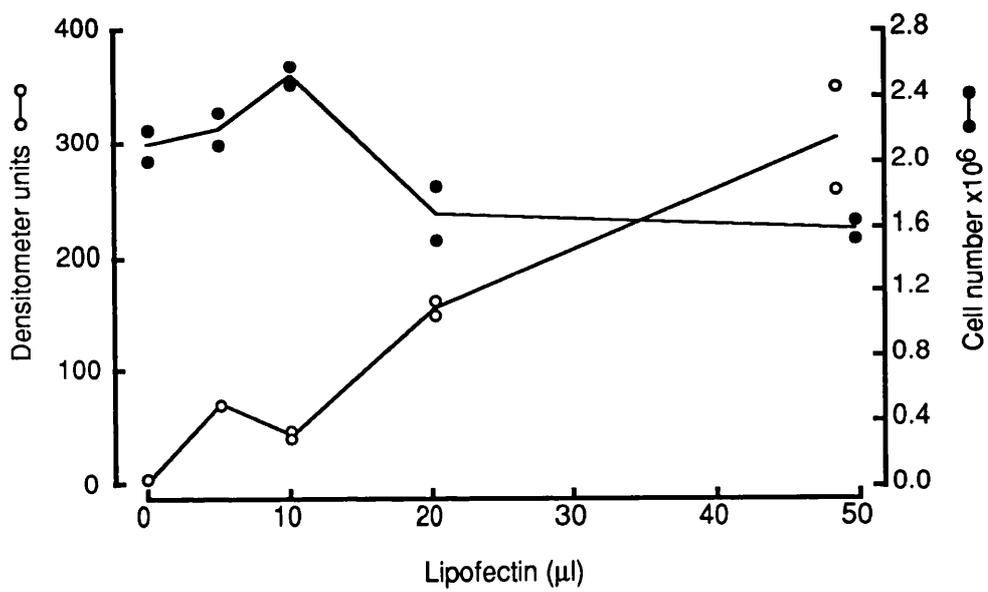


Figure 4.12. Optimisation of the amount of lipofectin (5, 10, 20 and 50 μ l, 1 mg/ml) for transfections. Cells were plated at 2.5×10^5 cells per 60 mm petri dish for determining CAT activity (○—○) and cell number (●—●).

over that observed with lipofectin (10 μ l) in this example, this was not reproducible and consequently in subsequent transfections, 10 μ l lipofection was used.

Effect of serum

The effect of foetal calf serum (FCS) on basal CAT activity using the pCAT.PTH700 construct was investigated. Cells were co-transfected with the pCAT.PTH700 construct (5 μ g), and β -galactosidase plasmid (5 μ g) using lipofectin reagent (10 μ l) as described above. Six hours after transfection, serum-free medium was replaced with DMEM containing 0, 1, 5 and 10% FCS in duplicate for 48 hours. Cell extracts were prepared for CAT and β -galactosidase activities.

In the absence of FCS, very low CAT activity was detected whilst maximum basal activity was observed with 5% FCS. CAT activity in the presence of 1% FCS was not different to that seen with 5% FCS. However, in the presence of 10% FCS, basal activity was reduced to 60% of that detected in the presence of 5% FCS, suggesting that FCS may have factors which have a suppressive effect on basal CAT activity. Subsequently, FCS at 2.5% in DMEM was used.

Optimisation of VDR co-transfection

A plasmid expressing VDR cDNA was co-transfected into OK cells since the level of receptors in these cells is very low. The VDR cDNA used was 2001 bp in length containing the coding and 3'-untranslated regions (Baker *et*

al. 1988) in the expression vector, pSVK3 (Pharmacia, Uppasala, Sweden; figure 4.13). pCAT.PTH700 construct (5 μg) and β -galactosidase plasmid (1 μg) were co-transfected with VDR/pSVK3 (4, 10, and 15 μg) with lipofectin reagent (10 μl) and the cells incubated for 6 hours as described earlier. 1,25(OH)₂D₃ at 100 nM or 0.1% ethanol in DMEM containing 2.5% FCS was added 6 hours post-transfection and cells were incubated at 37°C, 5% CO₂ for 48 hours. Cell extracts were prepared for CAT and β -galactosidase activities.

Co-transfection of VDR/pSVK3 with pCAT.PTH700 construct into OK cells gave maximal suppression of approximately 22% with 15 μg VDR/pSVK3 (figure 4.14). In the presence of VDR/pSVK3 (10 μg), CAT activity was suppressed by 5% and with VDR/pSVK3 (4 μg), a suppression of only about 3% was observed. In subsequent experiments, VDR/pSVK3 (15 μg) was co-transfected with pCAT.PTH700 (5 μg).

1,25(OH)₂D₃ dose response curve

In order to determine the optimum concentration of 1,25(OH)₂D₃ required for suppression, cells were co-transfected with pCAT.PTH700 (5 μg), β -galactosidase plasmid (1 μg) and VDR/pSVK3 (15 μg) with lipofectin (10 μl) for 6 hours. Serum-free medium was replaced with DMEM supplemented with 2.5% FCS containing 1,25(OH)₂D₃ at 0.1 nM to 100 nM in 0.1% ethanol or vehicle in duplicate. Plates were incubated for 48 hours at 37°C, 5% CO₂. Cell extract was prepared for CAT and β -galactosidase activities.

Suppression of CAT activity with 1,25(OH)₂D₃ was dose-dependent, 0.1 nM 1,25(OH)₂D₃ did not suppress CAT activity significantly, whilst 1 nM and

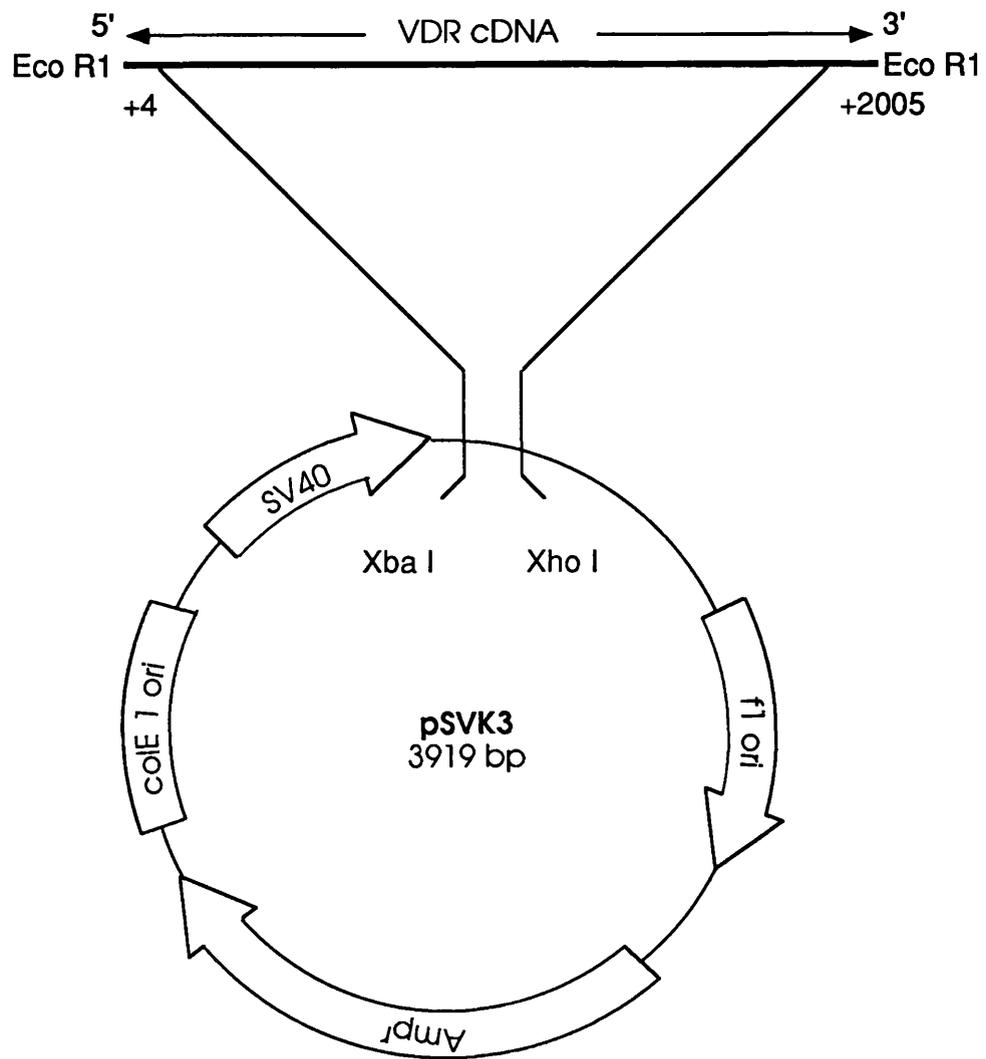


Figure 4.13 Subcloning of VDR cDNA (2001 bp) into the XbaI and XhoI sites of the expression vector pSVK3.

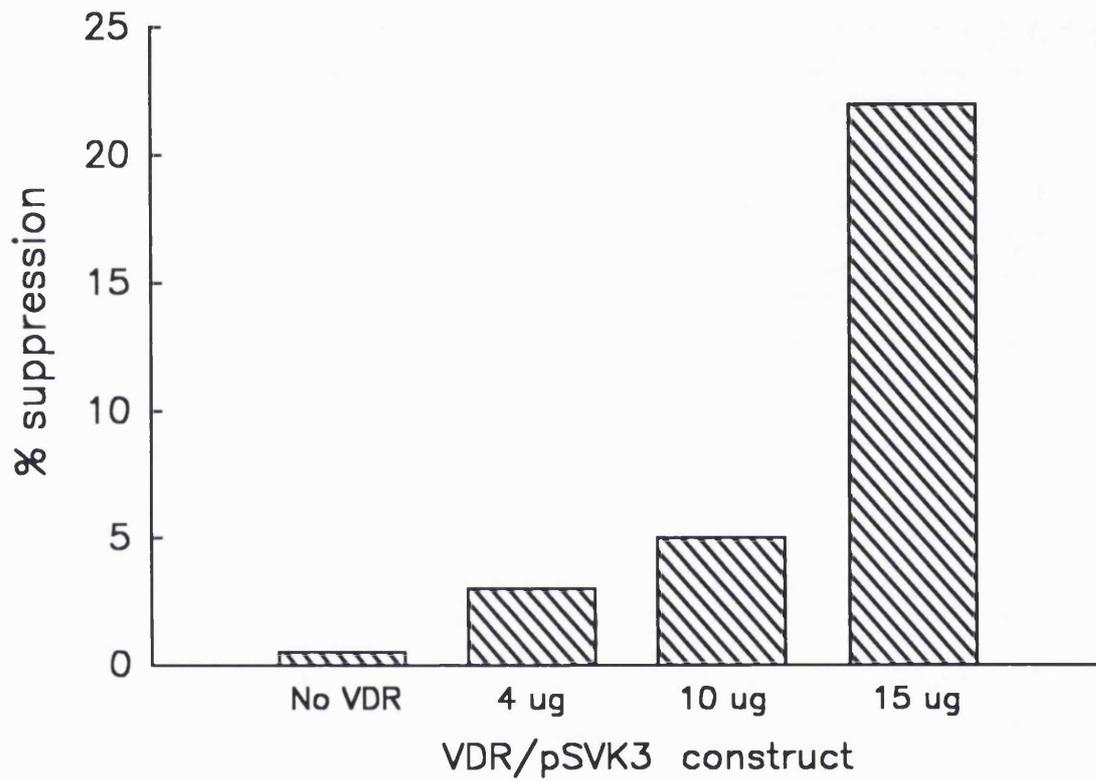


Figure 4.14. Optimisation of VDR/pSVK3 concentration for co-transfection with pCAT.PTH700 construct and β -galactosidase plasmid into OK cells. Lipofection was used at 10 μ g per dish and cells incubated for 6 hours at 37°C, 5% CO₂.

10 nM suppressed activity by 23% and 28%, respectively. Maximum suppression of CAT activity (38%) was observed when pCAT.PTH700 construct was co-transfected with 100 nM 1,25(OH)₂D₃ (figure 4.15). Thus in subsequent experiments, 1,25(OH)₂D₃ was used at 100 nM.

2. Transfection of PTH constructs

Following optimisation of conditions for transfection, plasmids pCAT.PTH700 (sense and antisense orientation), pCAT.PTH400, pCAT.PTH250 and pCAT.PTH100 (5 µg) were co-transfected with VDR/pSVK3 (15 µg) and β-galactosidase plasmid (1 µg) using lipofectin (10 µl) per 60 mm dish. Conditions were maintained as described earlier. 1,25(OH)₂D₃ at 100 nM was added after 6 hour incubation and medium was replaced on day 2 with fresh 1,25(OH)₂D₃ at 100 nM. Cells were plated in duplicate and cell extracts were assayed for CAT and β-galactosidase activities and protein in duplicate. All experiments were repeated a minimum of three times and CAT activity was related to protein.

The response of OK cells to 1,25(OH)₂D₃ changed depending on the number of passages and thus cells for transfections were used after being cultured for the same period. Thus, although the morphology of OK cells appears to remain the same, their responsiveness changes with age. Evidence for this is provided by Cole *et al.* (1989) who have reported that the effect of phorbol myristate acetate (PMA) on phosphate transport in parental and clonal subpopulations of OK cells was different even though morphologically they were similar.

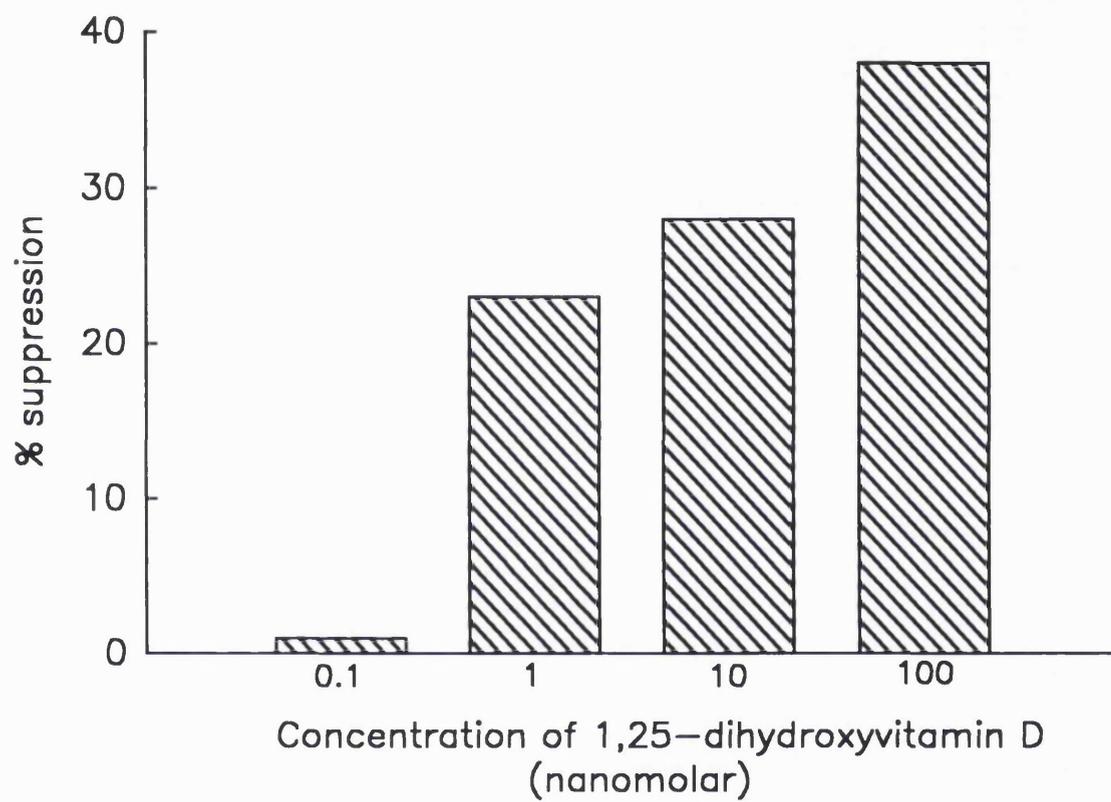


Figure 4.15. Dose response curve for $1,25(\text{OH})_2\text{D}_3$ (0.1 - 100 nM) on the suppression of CAT activity using the pCAT.PTH700 construct.

Transfection of pCAT enhancer plasmid (without insert DNA) into OK cells, revealed basal CAT activity which was not suppressed in the presence of $1,25(\text{OH})_2\text{D}_3$ (100 nM) (data not shown). Examples of autoradiographs showing CAT activity of the pCAT.PTH700, pCAT.PTH400, pCAT.PTH250 and pCAT.PTH100 constructs in the absence and presence of $1,25(\text{OH})_2\text{D}_3$ are illustrated in figure 4.16 and pooled data is shown in table 4.1. In the presence of $1,25(\text{OH})_2\text{D}_3$ (100 nM), CAT activity of the construct containing the region -668 to +50 bp (pCAT.PTH700, sense) was significantly reduced by $21.7 \pm 4.5\%$ (table 4.1). In contrast, the antisense construct did not mediate $1,25(\text{OH})_2\text{D}_3$ responsiveness suggesting that the response observed with the sense construct was specific. Furthermore, in the presence of $1,25(\text{OH})_2\text{D}_3$, CAT activity of pCAT.PTH250 and pCAT.PTH100 constructs containing the regions covering -668 to -452 and -451 to -348 bp was significantly suppressed by $27.0 \pm 9.0\%$ and $25.0 \pm 6.1\%$ of control, respectively, suggesting that these regions contain sequences responsive to $1,25(\text{OH})_2\text{D}_3$. However, deletion of the region covering -668 to -348 bp as in pCAT.PTH400 abolished suppression of CAT activity. There was no significant difference in the suppression of CAT activity mediated by the pCAT.PTH700, pCAT.PTH250 and pCAT.PTH100 constructs.

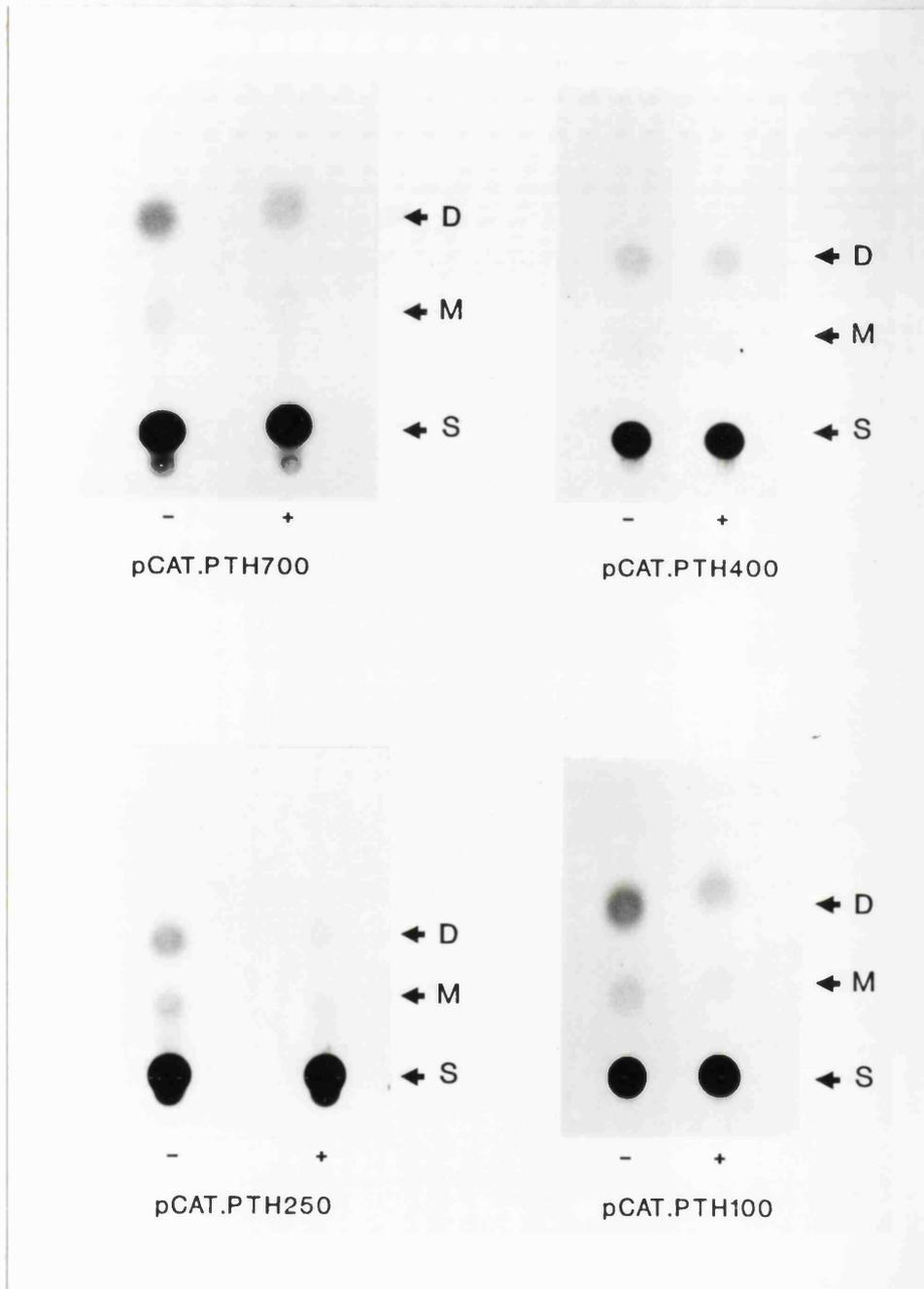


Figure 4.16. Examples of autoradiographs showing CAT activity in the absence (-) or presence (+) of 1,25(OH)₂D₃ (100 nM) of the pCAT.PTH700, pCAT.PTH400, pCAT.PTH250 and pCAT.PTH100 constructs.

PTH Construct	% Change
pCAT.PTH700	21.7 ± 4.5*
pCAT.PTH400	6.0 ± 2.8
pCAT.PTH250	27.0 ± 9.0*
pCAT.PTH100	25.0 ± 6.1*

Table 4.1 showing the percentage change in CAT activity mediated by the PTH constructs in the presence of 1,25(OH)₂D₃ (100 nM). Results are expressed as means ± SD (n=3). *p < 0.01 for comparison of pCAT.PTH700, pCAT.PTH250 and pCAT.PTH100 with their respective controls.

DISCUSSION

Data from these CAT assays demonstrated that $1,25(\text{OH})_2\text{D}_3$ (100 nM) suppressed transcription of the CAT gene initiated by the constructs containing the -451 to -348 bp fragment and the -668 to -452 bp fragment by $25.0 \pm 6.1\%$ and $27.0 \pm 9.0\%$ of control, respectively. CAT activity of the construct containing -668 to +50 bp fragment was reduced by $21.7 \pm 4.5\%$, however, the region containing the sequences -347 to +50 bp (pCAT.PTH400 construct), did not confer responsiveness to $1,25(\text{OH})_2\text{D}_3$. Furthermore, the presence of the -668 to +50 bp fragment in the antisense orientation did not induce CAT activity. These results are consistent with those obtained in Southwestern assays described in chapter 3 in which the -668 to +50, -668 to -452 and -451 to -348 bp fragments reacted with VDR immobilised on nitrocellulose filters, whilst the -347 to +50 bp fragment did not bind to immobilised VDR. The data from these functional assays confirm the presence of two regions containing sequences required for the effect of $1,25(\text{OH})_2\text{D}_3$ on PTH gene transcription. In addition, although the pCAT.PTH700 construct contains both putative VDREs, the suppression of CAT activity may be expected to be greater than that observed with the smaller fragments, however, this did not occur. One explanation for this observation is that the sequences flanking the two VDREs may be important in the interaction of VDR and VDRE possibly through conformational changes. In addition, it is possible that greater suppression did not occur in the pCAT.PTH700 construct due to the presence of sequences 3' to the VDREs (that is, -347 to +50 bp) which bind other factors thus attenuating the

suppressive effect of $1,25(\text{OH})_2\text{D}_3$ by, for example, preventing binding of VDR.

To show that the differences in suppression of CAT activity by these constructs was not due to methodological manipulations, the efficiency of transfection was assessed using β -galactosidase as an internal control which was co-transfected with the CAT constructs. The level of β -galactosidase activity in the basal and $1,25(\text{OH})_2\text{D}_3$ -treated cell extracts was approximately the same, with none detected in untransfected cell extracts, indicating minimal variation in the uptake of DNA into cells during transfection. In addition, plasmid DNA was introduced into OK cells using a liposome-mediated gene transfer technique which did not affect cell viability.

In general with steroid hormones, the presence of two response elements as seen in this study is not surprising, for example, two glucocorticoid response elements (GREs) have been identified in the rat tyrosine aminotransferase gene (TAT) in which the proximal element has no inherent capacity by itself to stimulate transcription whereas the distal element was capable of eliciting about one-third of the maximal response in CAT expression studies (Jantzen *et al.* 1987). When present in conjunction with the distal GRE, the proximal element synergistically enhanced glucocorticoid induction of gene expression. Cooperativity was also maintained when the GREs were placed upstream of a heterologous thymidine kinase promoter suggesting that no additional elements specific to the TAT gene are required for hormonal response. Thus, two separate but interacting domains constitute the functional GRE of the TAT gene.

More specifically in relation to $1,25(\text{OH})_2\text{D}_3$, the presence of at least two VDREs have also been identified, for example, in the human osteocalcin gene (Ozono *et al.* 1990), in which only the downstream element is able to confer $1,25(\text{OH})_2\text{D}_3$ responsiveness, whilst the upstream element is not essential for $1,25(\text{OH})_2\text{D}_3$ induction but enhances the response of the hormone. Deletion of sequences within the upstream element did not lead to a complete loss of hormonal induction, but exhibited 60-70% of the activity. However, deletion of the downstream element resulted in almost complete loss of activity (10%), indicating that this element was required for $1,25(\text{OH})_2\text{D}_3$ inducibility. Maximal levels of promoter activity were achieved when both elements were intact, suggesting that a functional synergism occurs between the two elements. The presence of two motifs is similar to the data obtained in this study on the bovine PTH gene, although synergism between elements was not observed. This may be because $1,25(\text{OH})_2\text{D}_3$ stimulates osteocalcin gene activity but suppresses transcription of the PTH gene, and it is perhaps not surprising that there is a difference.

Two similar regions that mediate $1,25(\text{OH})_2\text{D}_3$ responsiveness to the CAT gene have also been identified in the rat osteocalcin gene (Demay *et al.* 1990) in which only the downstream element is able to confer $1,25(\text{OH})_2\text{D}_3$ responsiveness. Further studies have revealed three motifs present within the downstream element that are critical for interactions with $1,25(\text{OH})_2\text{D}_3$ receptors (Demay *et al.* 1992a). Mutations of selected bases in the first two elements did not result in induction of CAT activity, whilst those in the third motif alone resulted in approximately a 50% decrease in gene activation. In

addition, no induction of CAT activity was observed when bases in the second and third motifs were mutated, suggesting that the first two motifs are critical for $1,25(\text{OH})_2\text{D}_3$ responsiveness and that the third motif may play a role in the interaction of the two upstream motifs with VDR.

In contrast to the two half-site arrangement of the osteocalcin VDRE and the putative bovine PTH VDRE described here, the studies of Demay *et al.* (1992b) on the human PTH (hPTH) gene have shown the presence of a single motif (AGGTTCA) in the region -113 to -107 bp which was found to confer $1,25(\text{OH})_2\text{D}_3$ responsiveness in cultured rat pituitary cells (GH_4C_1). A similar sequence is present in the bovine gene with one nucleotide difference (AGGATCA) within the region -108 to -102 bp. Despite the similarity of the sequence, the bovine PTH gene fragment -347 to +50 bp containing this region did not bind VDR in Southwestern assays as shown in chapter 3, and in addition, failed to induce CAT activity shown in this chapter. It is possible that VDR bind to the region -108 to -102 but with lower affinity than the two upstream elements described in the present study, with the result that the interaction was undetected in Southwestern or CAT assays.

The data from Demay *et al.* (1992b) does not exclude the possibility that other sequences further upstream in the hPTH gene interact with the receptor for a number of possible reasons. The first of these is that in the initial characterisation of the VDRE, the osteocalcin VDRE was used as a probe and fragments of the hPTH gene were used to compete for binding to VDR in gel shift assays. Fragments spanning the region -564 to +5 bp and -175 to +5 bp inhibited binding to the osteocalcin VDRE to approximately the same

extent ($87 \pm 0.9\%$ and $93 \pm 4.4\%$ respectively) and only the -175 to +5 bp fragment was characterised further in CAT assays. However, the selection of only the smaller fragment to study further was based on the assumption that since the two fragments inhibited binding to the same extent, the smaller fragment must contain the only VDRE. However, this does not allow for any positive or negative contributions on the interaction between VDR and VDRE from any flanking sequences. Therefore, to exclude any VDRE being present within the region -564 to -175 bp, it would have been interesting to use this fragment to show negative binding.

A potential problem of using the osteocalcin VDRE as a probe in gel shift assays, is that although there may be some similarities in the affinity for VDR, the VDRE in the PTH gene is not necessarily identical to that in the osteocalcin gene as $1,25(\text{OH})_2\text{D}_3$ suppresses transcription in the former but stimulates gene activity in the osteocalcin gene. In our recent experiments (data not shown), the ability of the putative bovine PTH VDRE in pBluescript plasmid to compete for VDR with a 800 bp region of the osteocalcin gene containing the VDRE in CAT plasmid was investigated. These results have shown that the PTH VDRE had no significant effect on the $1,25(\text{OH})_2\text{D}_3$ -induced CAT activity, suggesting that although the PTH and osteocalcin VDRE interact with VDR in vitro, it appears that in vivo they require different cofactors. Therefore, it is quite likely that formation of the VDRE-VDR-accessory factor complex occurs not only in a cell-specific, but also in a gene-specific manner. For example, an accessory factor (nuclear accessory factor, NAF) is thought to be necessary for high affinity interactions of VDR

with the human osteocalcin VDRE (Liao *et al.* 1990; Sone *et al.* 1991). Recently, Carlberg *et al.* (1993) have demonstrated that the two naturally occurring VDREs, namely that of the mouse osteopontin gene and the human osteocalcin gene, which are both up-regulated by $1,25(\text{OH})_2\text{D}_3$, are activated differentially, that is, in RXR-dependent and RXR-independent manner, respectively. These observations could be applicable to the present study in which VDR may also require factors such as RXR or NAF to form a heterodimer with the PTH VDRE.

The question of cell-specific factors may also provide a possible explanation for the differences seen in the human and bovine PTH studies, in which different cell lines were used. Demay *et al.* (1992b) transiently transfected a pituitary cell line, whilst in the study being presented here, a kidney cell line was used. These cells may possess different factors specific for each cell type which may be involved in down-regulation of the PTH gene and thus different results. Interestingly, Demay *et al.* (1992b) did not observe down-regulation of the hPTH gene in the rat osteosarcoma cell line, ROS 17/2.8, emphasising that different cells may have cell-specific factors.

Thus, data from CAT assays have confirmed the findings of chapter 3, in which two elements within the region spanning -485 to -452 and -451 to -348 bp upstream of the initiation site in the bovine PTH gene were shown to interact with VDR. The functional assays have demonstrated that these sequences are necessary for $1,25(\text{OH})_2\text{D}_3$ to suppress bovine PTH gene transcription.

CHAPTER 5

CONCLUDING REMARKS

The goal of this thesis was to study the regulation of the PTH gene by its two major modulators namely, calcium and $1,25(\text{OH})_2\text{D}_3$. Firstly, the possible sites of action of low calcium on PTH synthesis were investigated. Data from this study have demonstrated that preproPTH mRNA was associated with membrane-bound polysomes and low extracellular calcium (0.4 mM) increased this association. In addition, actinomycin D did not abolish the low calcium-induced rise in preproPTH mRNA levels but unexpectedly increased polysomal preproPTH mRNA levels in cells incubated in 0.4 and 1.0 mM calcium. Sucrose density gradient analysis demonstrated that there was no significant difference in polysomal size in the presence of 0.4 and 1.0 mM calcium and, therefore, unlikely to be due to changes in the rate of initiation and/or reinitiation relative to the elongation rate. Furthermore, there was no evidence of a pool of non-ribosomal mRNA. These results indicate that low calcium probably increases the number of times a RNA molecule is translated and thus increasing the apparent half-life of preproPTH mRNA associated with polysomes.

In the second section of the thesis, the binding sites for receptors for $1,25(\text{OH})_2\text{D}_3$ within the bovine PTH gene were identified. $1,25(\text{OH})_2\text{D}_3$ suppresses PTH gene transcription via its receptors, VDR, which bind to DNA sequences, vitamin D₃ response element (VDRE) in the 5'-flanking region of the gene between -485 to -348 bp upstream of the initiation site. Two putative VDRE have been identified within this region which were responsive to $1,25(\text{OH})_2\text{D}_3$ in functional assays indicating that the transcriptional effect of $1,25(\text{OH})_2\text{D}_3$ was mediated through these sequences.

These findings indicate that regulation of PTH synthesis occurs at multiple sites (figure 5.1). In addition to the well-known effects on secretion, low calcium regulates PTH synthesis at the translational level possibly by increasing the apparent half-life of preproPTH mRNA on polysomes. This, of course does not exclude the possibility that under certain conditions, for example, prolonged hypocalcaemia, low calcium also acts on gene transcription. With regards to $1,25(\text{OH})_2\text{D}_3$, these studies have shown that the effects of this hormone are mediated through the VDRE as shown in figure 5.1, to alter transcription of the gene. It is possible that there are additional effects as there is increasing evidence for post-transcriptional effects of $1,25(\text{OH})_2\text{D}_3$, for example, $1,25(\text{OH})_2\text{D}_3$ has been shown to destabilise c-myc mRNA in HL-60 cells (Mangasarian & Mellon, 1993). This may possibly be due to the increased levels of and/or increased sensitivity of mRNA to RNases.

Early studies of gene regulation were limited to investigating changes at the level of secretion by measuring hormone levels, until the availability of cDNA which has permitted the study of changes in mRNA levels at transcription using methods such as dot-blot assays and Northern analysis. However, close inspection of some of the results has revealed instances in which changes in the rate of transcription did not parallel changes in hormone synthesis and/or secretion, indicating post-transcriptional regulation.

Studies of post-transcriptional regulatory mechanisms have necessitated the use of additional techniques in addition to those used to study transcription, for example, mRNA stability, rates of initiation and ribosome transit times.

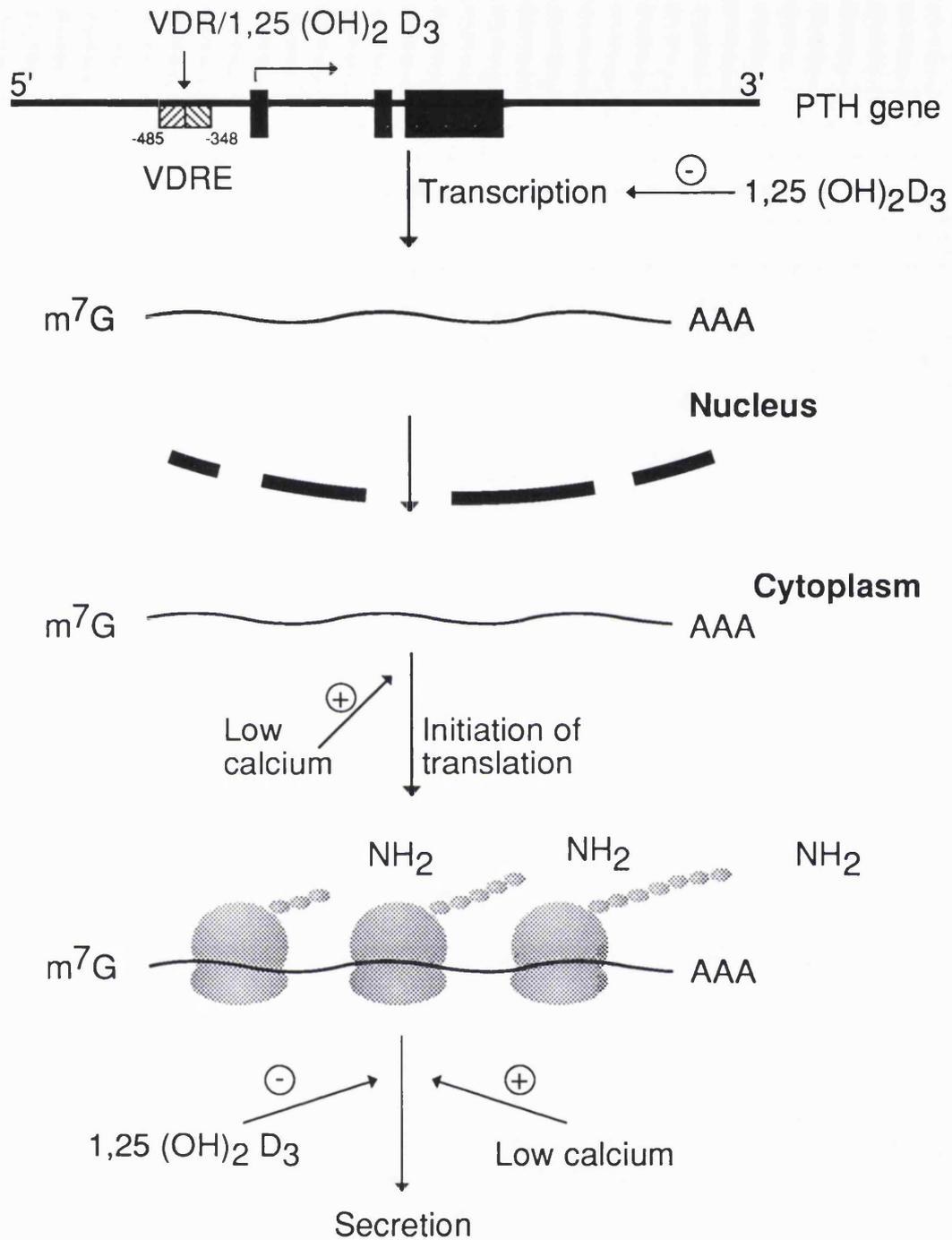


Figure 5.1. The possible sites of action of low calcium and 1,25(OH)₂D₃ on PTH synthesis. The VDRE shown as a cross-hatched box consists of two components: -485 to -452 and -451 to -348 bp.

Future studies on PTH gene expression would centre on investigations into the interactions between ribosomes, mRNA and proteins and would employ some of these techniques. As discussed in chapter 2, inhibition of transcription by actinomycin D potentiated the polysomal association of preproPTH mRNA suggesting that actinomycin D inhibits the synthesis of a protein which may be acting as a repressor of mRNA translation. For example, regulatory proteins binding to the 5'-untranslated region of preproPTH mRNA regulate ribosome:mRNA interactions, alternatively these proteins may bind to the 3'-untranslated region to alter half-life of mRNA at the polysome. Such proteins could be identified using, for example, UV cross-linking to mRNA and isolated using affinity chromatography before being sequenced. Conversely, essential sequences in the mRNA could also be identified. The interaction between mRNA and regulatory proteins may be dependent on the intracellular calcium concentrations. For example, low calcium may cause dissociation of a protein bound to the 5'-UTR of preproPTH mRNA thus increasing translation. It would be important to study such interactions in parathyroid adenomas in which PTH synthesis continues even in the presence of high concentrations of calcium.

As regards to the study of VDRE, these have been identified in a number of genes but the exact nature of their interaction with VDR and the possible involvement of other proteins such as NAF and RXR β remains unclear particularly since the mechanism of action may be different in for example, the PTH and osteocalcin genes since the former is down-regulated by 1,25(OH) $_2$ D $_3$ whilst the latter is up-regulated. These interactions could be

investigated further in for example, the *Drosophila* cell line (SL-3) which may be a good system since insects being less evolved do not contain endogenous mammalian factors which may otherwise interact with receptors so that the addition of accessory factors or vectors expressing receptors can be well-controlled.

Regulation at multiple sites has obvious advantages, for example, regulation at the translational level for instance, at initiation or reinitiation, would result in a more rapid synthesis of the protein rather than solely at transcription which would require new mRNA to be transcribed, processed, and transported to the cytoplasm where it is translated. An interesting example of how translation and transcription interact has recently been demonstrated in transgenic mice carrying the human insulin gene in which a two-fold increase observed in total RNA in the pancreatic B cells did not parallel the insulin levels and in addition, these mice responded similarly to a glucose challenge (Schnetzler *et al.* 1993). Their study demonstrated that although mRNA levels were raised, translation compensates for this increase, leading to a normal condition. Thus, it is likely that translation is the primary regulatory control mechanism in this instance.

In conclusion, the work presented in this thesis has elucidated some of the mechanisms of PTH synthesis. The sequences required for VDR binding have been identified in normal bovine parathyroid glands and this work could be extended to investigate these sequences in human parathyroid adenomas in which there is insensitivity to the effect of $1,25(\text{OH})_2\text{D}_3$. The reason for that is not clear; it might be due to the presence of mutations in this region or

another possibility is that it may be due to differences in the involvement and/or requirements of nuclear accessory factors which would need to be studied in human adenomas. RNA-binding proteins should be studied using cytosol and mRNA from adenomas in which there appears to be differential regulation at this level. In addition, it would also be interesting to study how the interaction between mRNA and binding proteins may be dependent on intracellular calcium concentrations. The calcium receptor has recently been cloned (Brown *et al.* 1993) and it would be possible to investigate whether the mechanisms described here are possibly dependent on the calcium receptor. The study presented here has demonstrated the multifaceted control of PTH synthesis and further investigations will help elucidate and understand the complex nature of PTH gene expression.

APPENDIX I

MATERIALS AND METHODS

General chemicals and reagents were obtained from either Sigma Co. Ltd (Poole, Dorset, U.K.) or B.D.H. Ltd (Poole, Dorset, U.K.), enzymes from Boehringer Mannheim (Lewes, East Sussex, U.K.), radiochemicals from Amersham International plc (Amersham, U.K.) and sterile plasticware from Falcon (Marathon, London, U.K.) unless stated otherwise.

Ligation reactions

Bacteriophage T4 DNA ligase (20 Units) was used to catalyse the ligation of blunt-ended DNA fragments into linearised dephosphorylated pBluescript II SK (+) or pCAT enhancer plasmids in the presence of 0.5 M Tris-HCl (pH 7.5) buffer containing 70 mM MgCl₂ and 10 mM dithiothreitol and 0.5 mM ATP (pH 7.5) at 4°C for 16 hours. For plasmid vectors and DNA fragments with cohesive ends, T4 DNA ligase (10 Units) was used in the above buffer.

For each ligation reaction, the number of pmoles ends/ μ g DNA for the plasmid and insert DNA was calculated using the following formula and the values are shown over the page:

$$\frac{2 \times 10^6}{660 \times \text{no. of base pairs}}$$

Plasmid/insert DNA	pmole ends/μg
pBluescript II SK (+)	1
pCAT enhancer plasmid	0.6
-451 to -348 bp	30
-451 to +50 bp	5
-347 to +50 bp	7.6
-668 to -348 bp	9
-668 to -452 bp	12
-100 to -2 bp	30

For blunt-ended ligations, a ratio of between 10-30 of insert DNA to plasmid DNA was used and for cohesive ends, a ratio of between 5-10 was used. Following ligation, competent cells (XL1-blue or HB101) were transformed with ligation mix (35 ng) as described below. 6-10 colonies were picked aseptically into 10 ml SOC or LB-broth media (prepared as described in Appendix II A) and plasmid DNA was isolated by small-scale preparation as described later.

Transformation of competent HB101 E.coli cells

Competent HB101 E.coli cells (0.2 ml) were mixed with pCAT enhancer plasmid containing insert DNA (30-40 ng) or control plasmid pBR322 (0.1 ng) diluted in 0.2 ml 5 mM Tris-HCl (pH 7.6), 0.1 M CaCl₂, 0.25 M KCl, 5 mM MgCl₂ and 10 mM RbCl (transformation buffer). Transformants were left on ice for 25 minutes and then transferred to 37°C for 5 minutes. Pre-warmed

LB-broth (1 ml) was added to each tube and incubated at 37° C for 1 hour without shaking. Transformation mixture (0.2 ml) was spread onto LB bacteriological agar plates containing 100 µg/ml ampicillin at 20°C and left for 1 hour until the liquid had absorbed. The plates were inverted and incubated at 37°C for 16 hours. The number of colonies obtained with control pBR322 plasmid were noted and the efficiency of transformation calculated. 10-12 single bacterial colonies were picked using a sterile loop into 10 ml LB-broth and used for small scale preparation of plasmid DNA.

Transformation of XL1-blue competent cells

XL1-blue competent cells were used for transformation of plasmid pBluescript II SK (+) which enables the selection of blue/white colonies. Some strains of E.coli such as XL1-blue cells have a modified lacZ gene, one that lacks a large segment lacZ' coding for the α -peptide of β -galactosidase. These mutants can synthesise the enzyme in the presence of a plasmid carrying the missing lacZ' segment of the gene. Thus, these cells enable the selection of colonies based on blue/white colour using 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) and isopropyl β -D-thiogalactoside (IPTG). X-gal is broken down by β -galactosidase resulting in blue colour in the presence of IPTG (an inducer of β -gal). Recombinant plasmids with disrupted lacZ' gene are unable to make β -galactosidase and remain white.

Plasmid pBluescript containing insert DNA (35 ng) or control plasmid pBR322 (0.1 ng) were mixed with freshly-thawed XL1-blue competent cells (100 µl) containing 25 mM β -mercaptoethanol (final concentration) in pre-

chilled tubes. The cells were left on ice for 30 minutes, heat-pulsed for 45 seconds at 42°C and placed on ice for 2 minutes. SOC medium (0.9 ml) pre-heated at 42°C was added to the cells and incubated at 37° C for 1 hour shaking at 225-250 rpm. To identify bacterial colonies that contain recombinant plasmids, 2% (w/v) X-gal (100 µl) and 100 mM IPTG were spread onto agar plates (40 µl). Transformation mixture (0.2 ml) and the control plasmid pBR322 diluted 1:40 in SOC medium were plated onto SOC agar plates containing 100 µg/ml ampicillin in 10 mM MgSO₄ solution. The liquid was allowed to absorb for 1 hour at 20° C before the plates were inverted and incubated at 37°C for 16 hours. To enhance the blue colour, the plates were stored at 4°C.

Transformation efficiency

The number of colonies obtained by transformation of SspI fragments in pBluescript using XLI-blue competent cells were noted and the efficiency of transformation was calculated from the control plasmid plate as shown below:

$$Efficiency = \frac{\text{colonies on control plate}}{\text{ng of pBR322}} \times \frac{10^3 \text{ ng}}{\text{ug}}$$

Typically, efficiencies of 2.0 - 3.2 X 10⁸/µg pBR322 were obtained.

Preparation of competent HB101 E.coli cells

Competent HB101 cells (10 µl) (Gibco BRL, Paisley, U.K.) were

transferred into 10 ml LB-broth and allowed to grow at 37°C with vigorous shaking for 16 hours. This subculture was used to inoculate 100 ml L-broth. Growth of the culture was monitored by measuring the optical density (OD) at 650 nm every 30 minutes for 2 hours at 37°C. Cells were allowed to grow until the OD reached 0.6, followed by centrifugation at 1500 g at 4°C, the supernatant discarded and the cell pellets resuspended in 5 mM Tris-HCl (pH 7.6), 0.1 M CaCl₂, 0.25 M KCl, 5 mM MgCl₂ and 10 mM RbCl containing 50% glycerol (storage buffer). Competent cells were stored at -70°C.

Extraction and purification of plasmid DNA

a. Small-scale preparation of plasmid DNA

Using aseptic techniques, 10 ml LB-broth or SOC medium containing 100 µg/ml ampicillin was inoculated with a single bacterial colony. At least 10-12 colonies were picked for analysis. The cells were allowed to grow at 37°C for 16 hours with vigorous shaking. 1 ml of each grow-up was removed, centrifuged at 8 800 g for 10 minutes, the pellet was resuspended in 500 µl storage buffer and stored at -70°C (as stock).

The remaining cells were harvested by centrifugation at 10 000 g for 30 minutes in an Eppendorf microfuge. The cell pellets were lysed in 25 mM Tris-HCl (pH 8.0), 10 mM EDTA and 50 mM glucose (200 µl), 0.2 M NaOH, 1% (w/v) sodium-dodecyl-sulphate (SDS) (400 µl) and 3 M potassium acetate (pH 4.3) (200 µl) and the cells were thoroughly mixed to ensure lysis. The lysed cells were centrifuged at 10 000 g for 10 minutes at 20°C followed

by the addition of propan-2-ol (0.6 volumes) and centrifugation for 5 minutes at 10 000 g at 20°C. The pellet was washed in 70 % ethanol and re-centrifuged for 5 minutes and the resulting pellet was dissolved in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA buffer (TE); boiled pancreatic RNAase A was added to a final concentration of 20µg/ml and incubated at 37°C for 30 minutes. Phenol saturated overnight with 20 mM Tris-HCl (pH 7.6), 1 mM EDTA and 0.1 M NaCl buffer (TEN) was mixed with an equal volume of isoamylchloroform (1:24) to give phenol isoamylchloroform (pic). Plasmid DNA was extracted with an equal volume of pic and the contents were centrifuged at 10 000 g for 2 minutes in a microfuge at 20° C. The aqueous layer was removed and re-extracted with pic and twice with an equal volume of isoamylchloroform. The aqueous layer was transferred to a fresh tube and the DNA was recovered by precipitation with 2 volumes of ice-cold 100% ethanol in the presence of 0.3 M sodium acetate (pH 5.2) at -20°C. DNA was recovered by centrifugation for 30 minutes at 4°C, washed with ice-cold 70% ethanol and dehydrated in ice-cold 100% ethanol. The pellet was dried under vacuum and redissolved in 50 µl TE buffer (pH 7.4). Test digestions were carried out using 3-5 µl of DNA with appropriate restriction endonucleases and analysed by agarose gel electrophoresis. The preparations which gave the expected fragments were grown in 500 ml growth media and analysed by large scale preparation as described below.

b. Large scale preparation and purification of plasmid DNA

Using aseptic techniques, 10 ml LB-broth/SOC containing 100 µg/ml

ampicillin was inoculated with 'stock' from small scale preparations and shaken vigorously for 16 hours at 37°C. This was used to inoculate 500 ml of LB-broth/SOC medium containing 100 µg/ml ampicillin and incubated at 37°C for 16 hours with vigorous shaking. Bacteria were recovered from the 500 ml cultures by centrifugation at 1500 g for 30 minutes at 4°C in a MSE high speed 18 centrifuge. The supernatant was discarded and the cell pellet was resuspended in 25 mM Tris-HCl (pH 8.0) containing 10 mM EDTA and 50 mM glucose (25 ml). NaOH (0.2 M), 1% (w/v) SDS (50 ml) was added and mixed thoroughly to ensure lysis, followed by 3 M potassium acetate (pH 4.3) (25 ml) and mixed. The lysed cells were centrifuged at 6000 g for 20 minutes at 4°C and the supernatant was filtered through cotton gauze into 250 ml centrifuge bottles. Propan-2-ol (0.6 volumes) was added, mixed well and centrifuged at 6000 g for 20 minutes at 20°C. The supernatant was decanted carefully and the pellet was washed with 70% ethanol at 20°C and the pellet was resuspended in 0.1 M Tris-HCl (pH 8.0), 2 mM EDTA buffer (5 ml).

Plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. Tris-HCl (0.1 M), 2 mM EDTA (pH 8.0) was added to the resuspended pellet until the total weight was 9 g. CsCl (1g/ml) and 10 mg/ml ethidium bromide solution (0.5 ml) were added, mixed and transferred to 16 X 76 mm Beckman polyallomer quick seal tubes. Centrifugation was performed at 202 000 g for 13 hours at 20°C in a near vertical rotor (NVT 65) in a Beckman L70 ultracentrifuge. The resulting lower band of closed circular plasmid was removed through the tube wall using a syringe. Tris-HCl (0.1 M), 2 mM EDTA buffer (pH 8.0) was added to a volume of 10 ml, followed

by 20 ml 100% ethanol at 20°C and centrifuged at 1500 g for 20 minutes. The pellet was washed with 100% ethanol and centrifuged again at 1500 g for 10 minutes at 20°C. The ethanol was discarded, the pellet was dried under vacuum and reconstituted in 0.15 M NaCl, 0.015 M tri-sodium citrate (pH 7.0) (1 X SSC) containing 100 µg/ml boiled bovine pancreatic RNAase A and incubated at 37°C for 1 hour. Plasmid DNA was purified by extracting with phenol:chloroform followed by isoamylchloroform and DNA was recovered by centrifugation as described above. The pellet was dried under vacuum and resuspended in 10 mM Tris-HCl (pH 7.4), 1mM EDTA buffer (TE) or distilled water. The yield and purity of DNA preparation was assessed by measuring the absorbance of a diluted sample at 260 nm and 280 nm.

Recovery of DNA fragments from agarose gels

In general, DNA fragments of 500-800 bp were separated on 1.5% or 2% agarose gels, whilst those less than 500 bp were separated on 0.8% agarose gels containing ethidium bromide (1.25 µg/ml). The DNA bands were visualised under UV light at 302 nm and the size of fragments were estimated by plotting DNA molecular size markers. Following separation on agarose gels, DNA fragments were recovered using the methods described below.

a. Gene Clean II kit

To purify DNA fragments greater than 200 bp in size, a Gene Clean II kit was used. Three volumes of sodium iodide solution were added to agarose gel slices and allowed to melt at 45-55°C; silica matrix (5-10 µl) was added

to each tube, the contents mixed thoroughly and incubated at 20°C for 5 minutes with occasional mixing. The silica matrix with DNA bound was pelleted for approximately 15 seconds at 10 000 g in a microfuge. The supernatant was decanted and the pellet washed three times in approximately 50 volumes of NaCl/ethanol/water wash. The washed pellet was resuspended in an equal volume of water and incubated at 45 - 55° C for 2 minutes. After centrifugation, the supernatant was collected and this step was repeated with an equal volume of water. The purity of DNA was assessed by measuring the absorbance of a diluted sample at 260 nm and 280 nm. The yield of DNA was calculated from the readings obtained at 260 nm.

b. Mermaid Kit

This method was used for purifying oligomers less than 200 bp in size. Three volumes of a concentrated solution of sodium perchlorate was added to agarose gel slices in microfuge tubes. Resuspended Glassfog (silica-based matrix) (8 $\mu\text{l}/\mu\text{g}$ DNA) was added and the contents mixed continuously for 10 minutes at 20°C. Following brief centrifugation at 10 000 g the supernatant was removed and the pellet washed three times with 300 μl of ethanol wash. The oligomer DNA was eluted from Glassfog by resuspending the pellet in a volume of water equal to the volume of silica added. After incubation at 45 - 55°C for 5 minutes, the contents were centrifuged for a minute. The supernatant was removed and the elution step repeated. An aliquot of the supernatant was measured at 260 nm and 280 nm.

Removal of 3' protruding ends

The exonuclease activity of T4 DNA polymerase was used to remove 3' protruding ends generated by digestion with PstI such as those in fragments -668 to -452 and -668 to +50 bp to yield blunt ends. In addition, pCAT plasmid digested with PstI was also blunt-ended. T4 DNA polymerase (14 Units) was used to catalyse the reaction in the presence of 2 mM solution containing dATP, dTTP, dCTP and dGTP at 12° C for 15 minutes. At the end of the reaction, the enzyme was inactivated by heating at 75°C for 10 minutes, purified by phenol:chloroform and DNA was recovered by centrifugation.

Filling recessed 3' ends

Recessed 3' ends were filled using the polymerase activity of the Klenow fragment of E.coli DNA polymerase I. After digestion with restriction enzymes, 1 Unit of Klenow/ μ g of DNA was used in the presence of dATP, dCTP, dTTP and dGTP at a concentration of 1 mM. The reaction was incubated for 15 minutes at 20°C, followed by inactivation of the enzyme at 75° C for 10 minutes and extraction with phenol:chloroform.

Dephosphorylation of DNA fragments

The terminal 5' phosphates of DNA fragments were removed before end-labelling with T4 polynucleotide kinase. The reaction was catalysed by calf intestinal alkaline phosphatase (0.8 mUnits/2 μ g DNA) in 0.5 M Tris-HCl (pH 9.0) buffer containing 10 mM MgCl₂, 10 mM spermidine and 1 mM ZnCl₂

at 37°C for 15 minutes at 56°C for 15 minutes. A second aliquot of 0.8 mUnits alkaline phosphatase was added and the incubation repeated at both temperatures. The reaction was terminated by heating at 68°C for 15 minutes in the presence of 0.5% (w/v) sodium-dodecyl-sulphate (SDS) and 100 mM Tris-HCl (pH 8.0) buffer containing 1 M NaCl and 10 mM EDTA. Dephosphorylated DNA fragments were purified by extraction with phenol:chloroform and recovered by alcohol precipitation.

Dephosphorylation of plasmid vectors

Linearised plasmid was dephosphorylated with calf intestinal alkaline phosphatase (CIP) to prevent reannealing of the vector. Linearised plasmids with blunt ends were dephosphorylated with CIP (14 mUnits) in 100 mM Tris-HCl (pH 8.3) buffer containing 10 mM ZnCl₂ and 10 mM MgCl₂ at 37°C for 15 minutes followed by another aliquot of CIP. Incubation was continued at 55°C for 45 minutes before the reaction was terminated with the addition of 5 mM EDTA (pH 8.0) at 75° C for 10 minutes. In order to dephosphorylate plasmids with cohesive ends, CIP (1 Unit) was used in the above buffer at 37°C for 30 min and the reaction was terminated by inactivation as described above. The dephosphorylated plasmid was purified by phenol:chloroform extraction, recovered by alcohol precipitation at -20°C and subsequently used for ligations. The amount of CIP used was based on the number of 5'-terminal phosphate residues present on pCAT plasmid which has 14 pmoles of 5'-terminal phosphate residues.

Endlabelling of DNA fragments with bacteriophage T4 polynucleotide kinase

Dephosphorylated DNA fragments were recovered by centrifugation and the resulting pellet was resuspended in 10 mM Tris-HCl (pH 9.0), 1 mM spermidine and 0.1 mM EDTA (10 μ l). T4 polynucleotide kinase (13.5 U/ μ g) was used to catalyse the transfer of 32 P from 32 P- α -ATP (9.25 MBq) to dephosphorylated DNA in labelling buffer. The reaction mixture was incubated at 37° C for 30 minutes and terminated by heating to 70°C for 10 minutes followed by the addition of 20 mM EDTA to stop the reaction. End-labelled DNA fragments were separated from unincorporated 32 P- α -ATP by centrifugation through Sephadex G-50 columns, purified by extraction with phenol:chloroform and recovered by centrifugation.

Multiprime labelling

Linearised denatured pPTHm29 cDNA (230 ng) was labelled with 32 P- α -dCTP (3.7 MBq) catalysed by the Klenow fragment of DNA polymerase I (4 Units) (Multiprime kit, Amersham International plc, Amersham, UK) to a specific activity of 10^8 cpm/ μ g. The reaction was performed in the presence of random hexanucleotide primer (2 μ M) and deoxynucleotides (100 μ M, dATP, dGTP and dTTP) in Tris-HCl buffer (pH 7.8) containing MgCl₂ and β -mercaptoethanol at 37°C for 30 minutes. Unincorporated dNTPs were removed by centrifugation through Sephadex G-50 columns.

Nick Translation

Chicken β -actin (1 μg) was labelled with ^{32}P - α -dCTP (2.6 MBq) by nick translation using a nick translation kit (Amersham International, plc) catalysed by DNA polymerase I (2.5 Units) to a specific activity of 10^8 cpm/ μg DNA. The reaction was performed in the presence of 100 μM dATP, dGTP and dTTP in Tris-HCl buffer (pH 7.8) containing MgCl_2 and β -mercaptoethanol at 15°C for 90 mins. The reaction was terminated by the addition of 0.05 M EDTA (final concentration). Unincorporated label was removed by centrifugation through Sephadex G-50 spin columns.

Protein Assay (Lowry method)

Alkaline solution (1 ml containing 2% Na_2HCO_3 , 2% NaK tartrate and 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) was added to 200 μl protein sample or BSA standard solutions (0 - 100 $\mu\text{g}/\text{ml}$). After incubation at 20°C for 10 min, 100 μl Folin's reagent diluted 1:4 was added and incubated at 20°C for 30 minutes. Absorbance was measured at 510 nm and the concentration of the proteins was determined from the standard curve. A linear relationship was seen within the range 0-100 $\mu\text{g}/\text{ml}$.

APPENDIX II

A: MEDIA

LB Broth (Luria-Bertani Medium)

Per litre:

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g

Sterilised by autoclaving

SOB Medium (per litre)

Bacto-tryptone	20g
Bacto-yeast extract	5g
NaCl	0.5g

Sterilised by autoclaving. Before use, 10 ml of a solution containing 1.0 M MgCl_2 and 240 mM MgSO_4 was added per litre.

SOC Medium

Identical to SOB medium, but containing 20 mM glucose (final concentration).

Bacteriological agar plates

12 g per litre bacto-agar was added to LB broth.

B. BUFFERS

1. TE

10 mM Tris-HCl (pH 7.4)
1 mM EDTA

2. TEN

0.1 M NaCl
20 mM Tris-HCl (pH 7.6)
1 mM EDTA

3. TAE (10 X) pH 7.0

0.4 M Tris
0.02 M EDTA
0.2 M sodium acetate

4. TBE (5 X) pH 8.3

0.445 M Tris
0.445 M boric acid
0.005 M EDTA

5. SSC (20 X) (pH 7.0)

3 M NaCl
0.3 M sodium citrate

6. Restriction enzyme buffers

i) Buffer A

33 mM Tris acetate (pH 7.9)
10 mM Mg-acetate
66 mM K-acetate
0.5 mM Dithiothreitol (DTT)

ii) Buffer B

10 mM Tris-HCl (pH 8.0)
5 mM MgCl₂
100 mM NaCl
1 mM β-mercaptoethanol

iii) Buffer M

10 mM Tris-HCl (pH 7.5)
10 mM MgCl₂
50 mM NaCl
1 mM Dithioerythritol (DTE)

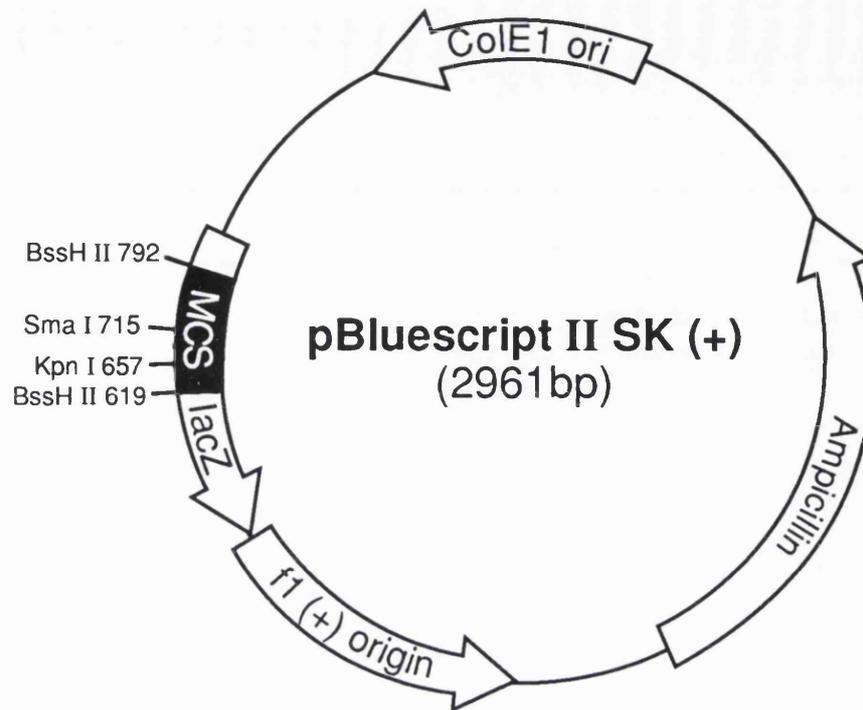
iv) Buffer H

50 mM Tris-HCl (pH 7.5)
10 mM MgCl₂
100 mM NaCl
1 mM DTE

7. DNA gel loading buffer

0.2% bromophenol blue
50% glycerol in TE (pH 7.4)

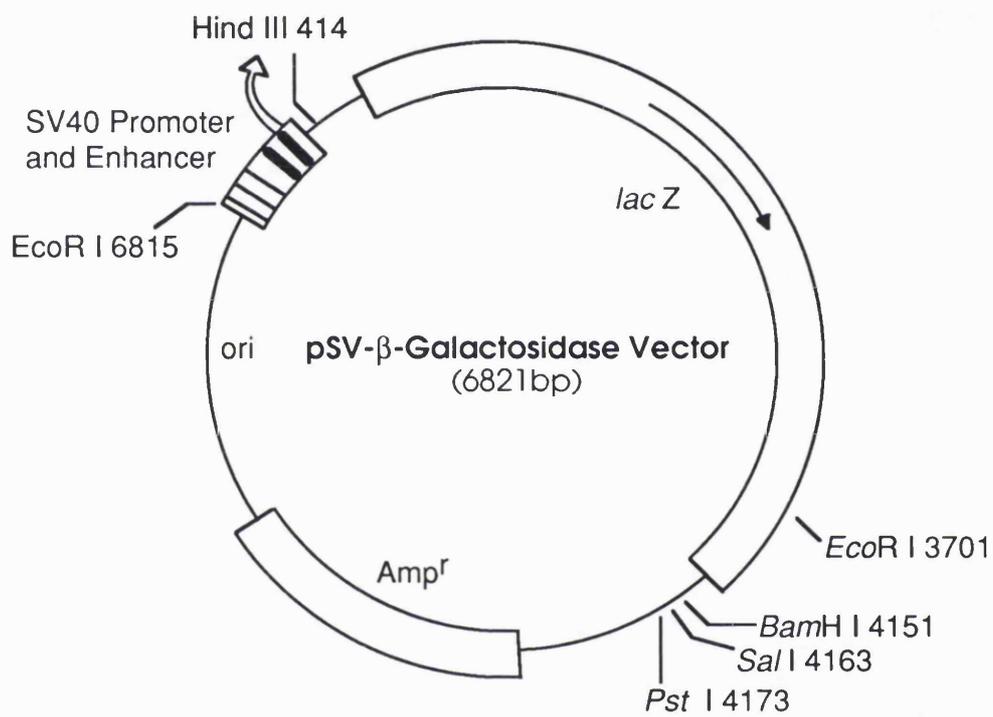
APPENDIX III: PLASMID MAPS



Plasmid contains the M13 - 20 primer just outside the MCS:

3' - T G A C C G G C A G C A A A T G - 5'

Map of plasmid pBluescript II SK (+) showing a lacZ promoter for blue/white colour selection and the multicloning site (MCS) containing restriction sites. Shown below the map is the M13 - M20 primer sequence which lies outside the MCS.



Map of the plasmid containing the *lacZ* gene encoding for β-galactosidase which was used as an internal control in transfection experiments. The SV40 early promoter and enhancer drive the transcription of the *lacZ* gene.

RESTRICTION SITE ANALYSIS

Shown below is the restriction enzyme site analysis performed on the -668 to -452 bp region of the bovine PTH gene:

<u>ENZYME</u>	<u>NO. OF SITES</u>	<u>POSITION (bp)</u>
ASU 2	1	-512
AVA 2	1	-535
BBV 1	1	-502
BSM 1	1	-479
DDE 1	1	-626
ECO 0109	1	-536
FNU 4H1	1	-502
HINF 1	5	-622 -608 -522 -508 -485
MBO 2	1	-514
MNL 1	1	-627
NLA 3	1	-618
NLA 4	2	-660 -536
PLE 1	2	-608 -522
PPU M1	1	-536
SAU 96	1	-535
SFA N1	3	-600 -542 -472
TAQ 1	1	-511

The following restriction sites do not appear within this region:

AAT 2	ACC 1	ACY 1	AFL 2
AFL 3	AHA 2	AHA 3	ALU 1
ALW 1	ALW N1	APA 1	APA L1
ASE 1	AVA 1	AVA 3	AVR 2
BAL 1	BAM H1	BAN 1	BAN 2
BCL 1	BGL 1	BGL 2	BIN 1
BSP 1286	BSP H1	BSP M1	BSP M2
BSS H2	BST E2	BST N1	BST X1
CFR 1	CLA 1	DRA 3	EAE 1
ECO N1	ECO R1	ECO R5	FNU D2
FOK 1	GDI 2	HAE 1	HAE 2
HAE 3	HGA 1	HGI A1	HGI C1
HGI J2	HHA 1	HINC 2	HIND 3
HPA 1	HPA 2	HPH 1	KPN 1
MLU 1	MSE 1	MST 1	MST 2
NAE 1	NAR 1	NCI 1	NCO 1
NDE 1	NHE 1	NOT 1	NRU 1
NSI 1	NSP B2	NSP C1	PFL M1
PST 1	PVU 1	PVU 2	RRU 1
RSA 1	RSR 2	SAC 1	SAC 2
SAL 1	SAU 1	SAU 3A	SCA 1
SCR F1	SDU 1	SFI 1	SMA 1
SNA 1	SNA B1	SPE 1	SPH 1
SSP 1	STU 1	STY 1	TTH111 1

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